Fertilization success and gamete viability of the Pacific oyster (*Crassostrea gigas*) across a salinity gradient.

Patrick Mooney

Degree Thesis for Patrick Mooney (Bachelor of Renewable Natural Resources)

Degree Programme in Sustainable Coastal Management

Tammisaari, 2016

BACHELOR'S THESIS

Author: Patrick Mooney

Degree Programme: Sustainable Coastal Management

Supervisors: Maria Kihlström and Ane T. Laugen

Title: Fertilization Success and Gamete Viability of the Pacific Oyster (*Crassostrea gigas*) Across a Salinity Gradient.

Date: March 18, 2016 Number of pages: 29 Appendices: (4)

Summary:

Anthropogenic climate change has, and will continue to, exert its effects on aquatic environments. Among these, changes in salinity levels, especially in high-precipitation regions such as the Swedish west coast, may affect the survivability and spread of some species. The Pacific oyster (*Crassostrea gigas*), an invasive species recently arrived in Sweden, is likely to be affected by decreases in the salinity content of coastal waters. In order to test this, a fertilization experiment was carried out to test both the longevity and viability of oyster gametes across a salinity gradient. Triple-parent crosses were run in five different salinities. These included the local environment's minimum, maximum, as well as below-minimum salinities. The results show that gamete exposure to hyposaline waters adversely affected their viability, represented by lower fertilization success. The proportion of fertilized eggs was not negligible at the lowest salinity, albeit still significantly lower compared to that of the other salinities tested. While still warranting further studies, these results suggest that the Pacific oyster is capable of reproducing in less saline waters than its current range in Sweden denotes. Because the invasion into Swedish waters is fairly recent, it is not yet known what the future spread of the species will be. Results from this experiment point towards a possible northward range expansion.

Language: English Key words: oyster, Crassostrea gigas, fertilization, invasive

---

Contents

1 Introduction

Climate change, the rapid alteration of the environment on a global scale, is mainly attributed to anthropogenic effects. Its effects can, aside from resulting in direct environmental degradation, indirectly lead to changes in the composition, structure, and distribution of many species. Some of the direct impacts on the biosphere include decreases in oceanic pH, warmer sea and air temperatures, reduced global albedo, shrinking glaciers and loss of permafrost, and altered precipitation patterns (IPCC 2014). The implications of a changing climate are vast and intricate, yet many of these changes are observable and quantifiable for a variety of species, across multiple phyla. Such changes encompass the indirect, or secondary, effects of climate change on Earth's biota.

Moreover, as climate change progresses it is predicted that that waters in many coastal regions will become both fresher and warmer. In the western coast of Sweden, these effects may impose a biological strain on the local fauna, including the invasive Pacific oyster (*Crassostrea gigas*). Having only recently arrived in Sweden less than 10 years ago (Dolmer et al. 2014), this species has been able to settle and establish populations throughout the west coast of the country, rapidly expanding its range likely as a result of warming waters. Understanding C. gigas' response to hyposaline waters will provide us with a better understanding of the future of this species in Northern Europe.

One measure to test the likelihood of a species surviving in a novel aquatic environment is to quantify the efficiency of fertility, or the fertilization success rate. As *C. gigas* reproduces externally, its gametes, and if fertilization occurs, embryos and larvae, are exposed to the environment in which they are released in. Testing the effect of salinity on this invasive oyster species can therefore be measured by the fertilization success of the species in different salinities. Gamete viability, if sperm and eggs are able to combine to form embryos, and longevity, how long oyster sperm and eggs survive in water to fertilize, is expected to be highest in water with salinities similar to the species' optimal salinity range. Yet in light of climate change, the Pacific oyster's range in Northern Europe is expected to change.

In the summer of 2015, as a part of the oyster research group at the *Sven Lovén Centre for Marine Sciences* in Tjarnö, Sweden, I conducted an experiment testing the fertilization success of the Pacific oyster. The contributions from this thesis serve two purposes: to determine gamete longevity and to test the effects salinity has on fertilization success.

1.1 Hypothesis

The fertilization success of the population of the Pacific oyster (*Crassostrea gigas*) will decline as water salinity is decreased, away from the population's optimal range.

1.2 Aims

1) Determining oyster gamete longevity after extraction from gonads
2) Testing gamete viability across a range of salinities

1.3 Approach

The main goal of this experiment was to test the combined effects of salinity and time on fertilization success. In order to do so, one population of Swedish oysters was used. Triple-crosses (3 males and 3 females) fertilizations were carried out; with each trial containing five different salinities and spanning a total of eight hours. After each time period eggs were counted and the proportions of fertilized against non-fertilized eggs were calculated.

2 Climate Change

In aquatic environments, marine organisms face three large hurdles imposed by climate change. First, ocean acidification, resulting from Earth's water bodies acting as carbon sinks, sequestrating carbon dioxide as bicarbonate ions, which in turn can harm sensitive organisms by impairing calcification. Corals, especially with regards to their mutualistic algae, are sensitive to lower pH, and massive die-offs are seen worldwide. A second issue is the increasing ocean temperatures, especially on surface waters. The latest IPPC report (2014) indicates an increase in temperature for global surface waters, those to a depth of 75 meters, of 0.11^{o} C per decade, from 1971 to 2010. Temperature tolerance varies greatly among species. Some are more robust and are able to survive in warmer waters, while other species are more sensitive. One survival mechanism for marine species is to shift their ecological range towards cooler waters. Additionally, since the bulk of fertilization in the oceans occurs externally, gametes, as well as early developmental stages will be at greater risk –as they tend to be less tolerant to temperature changes, as well as to changes in other abiotic factors.

The third major outcome of climate change is the alteration of water salinity levels, both on a local and global scale. Locally, regions of high evaporation rates, e.g the Mediterranean Sea, have

become saltier, while in regions of low salinity, such as coasts and estuaries, levels have become fresher because of increased precipitation and runoff. Globally, salinity levels have decreased (IPCC 2014). Melting glaciers and Arctic and Antarctic ice sheets pose an additional threat to ocean salinity levels. Higher global temperatures will continue to induce, and perhaps accelerate, glacial melting, resulting in increased freshwater runoff into the sea. This is likely to not only affect hydrological processes, but interfere with biological processes as well.

In areas of increased runoff and precipitation, decreased salinity concentrations can be stressful for marine organisms in two main ways. First, the ion concentration gradient between organisms and their environment will change, forcing a shift in osmoregulation in many species, which may be biologically costly for them. Additionally, even if adult organisms are able to withstand these changes, the more fragile and sensitive gametes –eggs and sperm- and early developmental stages are more susceptible to variations in the environment, since they lack the specialized regulatory mechanisms they would develop once they reach adulthood. Therefore, it is expected that in regions where salinity levels are altered, mainly as a result of climate change, organisms living there will be affected.

3 The Pacific oyster

The Pacific oyster (*Crassostrea gigas*) is a bivalve mollusk endemic to Japan. Mainly as a result of various intentional introductions for aquaculture practices, it has established populations throughout the world, where it is either prized for its commercial value or regarded as an invasive species and a nuisance. As recently as 2006, no established populations were known in Sweden, but since then *C. gigas'* range has expanded from the south of Varberg (57^{o}N) to Stromstad (58.9^{o}N) in the western coast of the country (Dolmer et al, 2014). This rapid expansion has led to numerous studies aiming to better understand the reasons behind the successful invasion, origins, as well as future expansion of the Pacific oyster.

Because of its importance in aquaculture, the Pacific oyster was introduced into Europe in the 1960's (Grizel 1991). At the time many thought *C. gigas* would not be able to establish populations in Europe due to the warmer water temperatures, yet the oysters did the exact opposite. Not long after, viable populations were found throughout the western coast of the continent. The recent and rapid expansion of *C. gigas* across Northwestern Europe has prompted extensive studies attempting to understand the reasons behind this spread, as well as predicting the future distribution of this

species. At the *Sven Lovén Centre for Marine Sciences* in Tjarnö, Western Sweden, a variety of experiments are underway with the aim to describe this species' tolerance to changes in environmental abiotic factors.

4 Oyster life cycle

Crassostrea gigas is a protandrous hermaphroditic species, usually developing first as males, yet being able to switch sex to females in short periods of time (Dolmer et al, 2014). When environmental conditions are unfavorable, mainly when food is scarce, females may return to being male. Food abundance, therefore, tends to favor female-dominant populations. Not only are the oyster shell valves irregular in size and shape but wild oysters also show a variety of morphological differences (Nehring 2006). Farmed oysters, on the other hand, have an elongated, distinct and predictable shape. When in distress, they are capable of completely sealing their shells when in the presence of predators, poor weather conditions, or when facing extended air exposure. Shells of adults are tough and their edges are sharp enough to cause lacerations on the skin, which can easily occur in beaches people frequent.

The size of adults varies between 50 and 80 mm within the first year post-settlement up to 200 mm or even 400 mm in older individuals (Nehring 2006, Dolmer et al, 2014). Pacific oysters can live well over 20 years. By the summer following settlement, oysters normally develop mature gonads, allowing for reproduction to take place (Toost 2010, Dolmer et al, 2014). Gametogenesis is usually triggered at temperatures above 10 °C and in salinities between 15-28 PSU (practical salinity units) (Dolmer et al, 2014). Reproduction occurs externally, though mass spawning, in the months of July and August, occurring usually at around 20 °C and at a salinity range of 23-36 PSU (Cardoso et al, 2006, Nehring 2006). Once gametes have been released into the water, fertilization must occur in a 10 to 15-hour margin (Dolmer et al, 2014). After fertilization has taken place, oyster embryos develop into planktonic larvae. They can survive in this stage for up to four weeks, and may travel, with the aid of water currents, hundreds of kilometers from their place of origin before settling on a hard substrate. Optimal conditions for settlement are at temperatures (in Celsius) and salinities (PSU) higher than 20 (Wrange et al, 2010). Following settlement, larvae undergo metamorphosis, developing into sessile Spat. Spat grow and mature rapidly into reproductively-active adults (Troost 2010).

5 Habitat (origin and distribution)

The Pacific oyster is now found in all regions of the world, with the exception of the Arctic and Antarctic circles. It is either invasive, cultivated, or both, in North and South America, the western coast of Europe, the British Isles, Africa, Oceania, and most recently in Scandinavia (Troost 2010, Dolmer et al, 2014, and Fabioux et al, 2002). Often intentionally introduced for aquaculture practices, eg: France (Cardoso et al, 2006) Holland (Dolmer 2014), and Sweden (Troost 2010), oysters were not expected to reproduce successfully in environmental conditions differing from their native range. Temperatures in Western Europe were thought to be too warm for oysters to reproduce, while in Scandinavia low winter and summer temperatures would be enough to inhibit fertilization. Yet, the current distribution shows otherwise. Found as far north as 60 °N in Norway, and having well-established populations across Europe's western seaboard, *Crassostrea gigas* appears to be a successful invasive species.

C. gigas is an intertidal filter-feeding species with a preference to estuarine waters (Miossec et al, 2009), yet it is still able to thrive in a wide range of littoral environments. Dolmer et al (2014) describe the main habitat characteristics where these oysters are found in Sweden: shallow (0-2m depth) waters, usually attached to other oysters or mussels; and well-protected to semi-exposed beaches, as well as those with significant water circulation. And while planktonic larvae prefer settling on hard substrates, often on other oysters or local mussels, they are also capable of attaching to small pebbles or shell shards found on sandy beaches.

6 Environmental tolerance and evolutionary advantages

Seeing that previous expectations of oyster settlements in Europe have been erroneous (Hollander et al, 2015 & Cardoso et al, 2006), it is important to consider the characteristics that have allowed this species to thrive and expand rapidly throughout the continent, as well as the implications further expansions may entail. Different evolutionary adaptations have enabled *C. gigas* to spread rapidly and become established all over the world. Below is an overview of these strategies.

6.1 Temperature

Even though early developmental stages are not as resistant to extreme environmental conditions as adults, the presence and reoccurrence of *C. gigas* in Scandinavia, where surface waters can easily

freeze, to the Mediterranean, where water temperatures can exceed 30 °C in the summer, indicates that early life stages only play a minor factor in oyster range expansion. An experiment by Child and Laing (2008) showed high spat mortality (>95%) after 3-7weeks at 3 °C, yet noted that all spat survived in temperatures of 6 and 9 °C. On the other end of the thermal tolerance spectrum, extended exposure (5 weeks) to 32 °C water has a significant impact (survivability <50%) on spat compared to (>88%) at 23-29oC (Florens-Vergara et al, 2004).

Even with these apparent reproductive constraints, *C. gigas* has established colonies across Scandinavia. Troost (2010) suggests that in 2006 a mild winter and warm summer may have helped ease the recent invasion into northern Scandinavia. But not only has the species colonized far into Northern Europe, but according to Wange et al (2010) and Laugen et al. (2015), *C. gigas'* range may continue to expand, as the species has yet to reach its environmental limits, and may continue moving northward.

Adults are even more resistant to extreme environmental conditions than the younger life stages. In the regions where established Pacific oyster populations are found, sea surface temperature and air temperature averages between 14 - 28.9 °C, and 15 - 31°C respectively, in the warmest month and -1.9 - 19.8 °C, and -23 – 14 °C respectively, in the coldest month (Dolmer et al, 2014). In an experiment by Strand et al (2011), oysters were exposed to temperature of -22 °C over a period of 72 hours, simulating an exposure to cold air in Scandinavia during wintertime. There was an overall survivability of 50% after a 24-hour exposure. Populations that underwent thermal acclimatization prior to the cold treatments had a higher survival rate, likely as a result of a preparation for wintertime, than those that did not.

6.2 Salinity

C. gigas is a euryhaline species, as it is capable of living in salt and freshwater environments. It is also an osmoconformer species, by maintaining internal osmotic levels similar to that of its environment. These traits enable it to survive and acclimate to a wide range of salinities. When exposed to changes in water salinity, it is capable of acclimating to the new environmental conditions by altering its hemolymph, or blood, chemistry; yet persistent and acute salinity changes may be lethal (Knowles et al, 2014). An alternative strategy this oyster employs is to endure environmental perturbations by clasping its shells shut. By doing so, respiration and feeding are suspended, but at the same time water is not able to enter the organism and disrupt its internal osmotic balance. The Pacific oyster can be found in freshwater, below 5 PSU, and well above oceanic salinity of 35 PSU (Dolmer et al, 2014 & Nehring 2006). Likely as a result of the toll the

synergistic effect of low water temperatures and low salinities has on oyster physiology, Swedish populations of *C. gigas* are limited to waters with salinities above 20 PSU (Wrange et al, 2009).

C. gigas is found at salinities well below 10 PSU (Nehring 2006), yet in Sweden populations are limited to salinities above 20 PSU (Dolmer et al, 2014). This may be a result, mainly, of the combined effect of salinity with temperature, seeing that decreases in pH and predation do not have significant effects on oyster mortality or on their distribution. Gamete tolerance to low salinities in Swedish oyster populations may add weight to the hypothesis (Wrange et al, 2009) that the range of *C. gigas* in Northern Europe is likely to continue to expand.

Many other environmental parameters affect the dispersal and survivability of this species. Aside from salinity, other factors which could influence, negatively or positively, include: increased surface water temperatures, intensity of predation, ocean acidification, and, most importantly, the interaction between some or all of these parameters.

7 Environmental and community effects

R-selected species, like the Pacific oyster, are able to reach maturity quickly and have high fecundity and survival rates. Furthermore, a wide variety of characteristics have made *C. gigas* a successful invasive species. These are outlined in *Table 1*. alongside their respective environmental implications. Many of the characteristics described below can be attributed to most bivalves. The purpose of the column describing the significance of each trait is to denote those qualities that have given *C. gigas* an advantage over other members of the class Bivalvia.

Table 1. Characteristics enabling *C. gigas* to be an effective colonizer species, with an emphasis on Swedish populations. Extracted from Troost (2010). Some characteristics omitted for space. Additional information was obtained from the background of this paper.

Category	*Trait*	*Implications*	*Significance*
Life cycle	Rapid sexual maturity and growth	Reproductive age is reached the year following settlement, leading to a new generation of reproductively active individuals each year. Limited exposure, as larvae and spat, to predators (mainly fish).	Moderate
	High fecundity	Strong competition with native mussel. *C. gigas*: 50-20 million eggs/spawning/female *Mytilus edulis*: 5-12 million.	Moderate
Environmental tolerance	Broad habitat	Generalist, and its range is still expanding. Found in Asia, Africa, North and South America, North and Western Europe, and Oceania.	Moderate
	High abiotic tolerance	Can survive in warm Mediterranean waters and freezing temperatures of Sweden and Norway. Range still expanding.	Moderate
	Phenotypic plasticity	Resilient species, even under harsh environmental conditions. Ease of recolonization after significant die-offs.	High
Interactions	Ecosystem engineering	Effective at reef formation on most hard substrates, especially other oysters –dead and alive – as well as on *M.edulis* shells, thus increasing the likelihood of continual settlement. Reefs are tough and well cemented; they also offer protection against predators.	High
	Lack of natural predators.	In Sweden it has no main predators. Herring gulls have been seen occasionally feeding on oysters, yet no significant oyster mortality is observed. Sea stars and shore crabs are being studied as potential predators. Low predation may lead to a reallocation of resources from predator deterrence to growth and reproduction (Cadée 2001).	High
Colonization	Ease of dispersal	Fertilized eggs and planktonic larvae are able to travel long distances, well over 100km, before settling and colonizing. Northern populations tend to produce more, yet smaller eggs, allowing for delayed larval development, enabling larvae to travel longer distances (Cardoso et al, 2006 & Moissec et al, 2009).	Moderate

8 Fertilization

Following gametogenesis, oocytes are stored in female gonads in an osmotically inactive state, allowing the oysters to carry a larger number of them prior to spawning. Once released into the surrounding waters, the eggs absorb water, through osmosis, and become turgid. Work by Stephano et al (1988) showed that eggs are viable shortly following spawning, but the risk of polyspermy (fertilization of a single egg by multiple sperm, preventing, by inhibiting, embryogenesis) is highest at this point. The likelihood of polyspermy in the Pacific oyster decreases over time. Additionally, resistance to polyspermy is significantly higher at 1-1.5 hours after oocytes have been allowed to incubate in seawater. Preliminary trials from my experiment pointed to polyspermy as a likely cause of minimal fertilization, since the sperm concentrations that were used were 3-4 times higher than in the experiment. Sperm concentrations for optimal fertilization success range between 5,000 and 10,000 cell/ul (Havenhand and Shlegel 2009). *C. gigas* also appears to have variable reproductive success, as a result of inconsistent gamete quality (Song 2009). Furthermore, Song outlines factors to consider when conducting fertilization experiments with Pacific oysters: fertilization occurs within 10 minutes of gamete contact, high (>5000 cell/ml) oocyte concentrations decrease total fertilization, and the highest yield is achieved with sperm to egg ratios between 100:1 and 500:1.

8.1 Determining gamete longevity

Fertilization in oysters occurs externally. For Pacific oysters in the wild, successful fertilization must happen within 10-15 hours after spawning (Nehring 2006). Environmental factors, such as temperature and salinity, can have an impact on this timeframe. Therefore, this experiment aimed to determine the effects of both salinity alone and the combined effects of time and salinity on fertilization success. Large salinity fluctuations may disrupt the osmotic balance in gametes, rendering them inviable. pH, on the other hand, has been shown not to have a significant effect on sperm motility (Havenhand & Shlegel 2009) and on early larval stages (Ko et al 2013).

9 Summary

Crassostrea gigas is an oyster species found throughout the world, where it has established populations in coastal and estuarine environments. It is tolerant to a variety of environmental factors, making it a successful invasive species. In Scandinavia it has undergone a rapid range expansion since the first confirmed settlements in 2006, and thus far it appears its range might

continue to increase. Alongside information of this species' life history, environmental tolerance, and invasive traits, determining the effects expected salinity changes will have on gamete longevity and fertilization success will provide us with a more thorough understanding of the species' future in Sweden and Northwestern Europe.

10 Methodology

In this experiment only the earliest life stages, from gametes to embryos, were utilized. Any observed effects would solely indicate sensitivity to salinity on the most vulnerable stages of development, thus suggesting a potential limiting factor of further dispersal by *C. gigas*. Moreover, due to time limitations, only sperm concentrations were standardized. Oocyte concentrations were maintained constant only in quantity, but not in egg density. Additional work on environmental effects on oyster reproduction is being carried out by other members of the research group. Tasks were divided, and different factors were analyzed in order to maximize the scope of the project. My contribution to the group is limited to one variable, salinity, on fertilization.

Oysters (>70 mm length) were collected less than three days prior to gamete extraction at a rocky beach near the *Sven Lovén Centre for Marine Sciences* at Tjarnö (58.879 °N, 11.140 °W), Sweden. Once inside, the oysters were kept in a ~1x1m^2 plastic box, with a constant flow of surface water from outside the laboratory. Oysters were, therefore, maintained at similar temperature and salinity conditions from whence they came (17.5ºC +/- 0.9, 26.8 PSU +/- 1.4). All the filtered fresh water (FFS) and filtered seawater (FSW), which ranged between 34 and 32.5 PSU, used to prepare the samples was kept at room temperature (21 °C +/- 1 °C).

Each trial-day began with gamete extraction. One by one, small (~3 mm diameter) holes were made on the shell of oysters with an electric drill until gametes were found. This was done until three females and three males were identified. Gametes were extracted with glass pipettes, and consequently distinguished as eggs or sperm under a microscope. Egg stocks were then transferred into three individual 15 ml Eppendorf tubes, and sperm into three different 2 ml Eppendorf tubes. In order to prevent the sperm from swimming, thus expending their limited ATP reserves, as tested by Havenhand and Shlegel (2009), tubes with sperm were kept in ice immediately following extraction. Most oysters survived and were, at the end of each day, released back into the sea.

Oyster eggs are stored in gonads in an osmotically inactive state, thus limiting space utilization inside females (Havenhand, pers. comm, June 2015). Consequently, eggs were placed in FSW one hour prior to the start of the experiment allowing them to become turgid and active. In order to minimize hyposaline shock, eggs were initially placed in 5 tubes with water of the highest salinity, then gradually fresh water was added until appropriate salinities were achieved (see appendix 8.1 for dilution table). After an hour, 1.35 ml egg solution was added into each of 75 tubes, subdivided into 5 salinities, with three sub-samples, for 5 time intervals.

Preliminary trials showed minimal fertilization, possibly due to polyspermia. To avoid this, sperm densities were determined as described by Havenhand and Shlegel (2009), and optimal sperm concentrations, between 5,000 and 10,000 cells/µl were used. Additionally, eggs were allowed to develop in FSW for an hour, which in turn decreased the probability of polyspermy occurring (Stephano and Gould 1988). In order to have diluted sperm solutions for simpler and more accurate distribution, sperm from all three males were transferred into five tubes containing water (6 ml) of the different salinities. These sperm solutions were then immediately added to every egg-containing tube for fertilization. Fertilization was allowed to occur in a span of two hours. In Song et al (2009), fertilization occurred within 10 minutes of gamete mixing. Tubes were kept at a constant temperature (21 °C +/- 1 °C), and in an inclined, slightly elevated from horizontal, position to prevent egg agglomerations. Failing to do so would have limited sperm contact with eggs at the bottom of the tubes.

A protocol had already been constructed by Havenhand, based on his previous work (pers. comm, June 2015). After a few trial runs it became clear that the current experimental setup was not leading to any results, likely as a result of polyspermy and gamete incompatibility. Therefore, after utilizing optimal sperm concentrations based on work by Havenhand and Shlegel (2009), trying a wide combination of egg concentrations, and various number of crosses, a more accurate protocol was developed (Appendix 8.3). Yet, because of time constraints, not all factors were standardized. Egg concentrations, for example, were highly variable

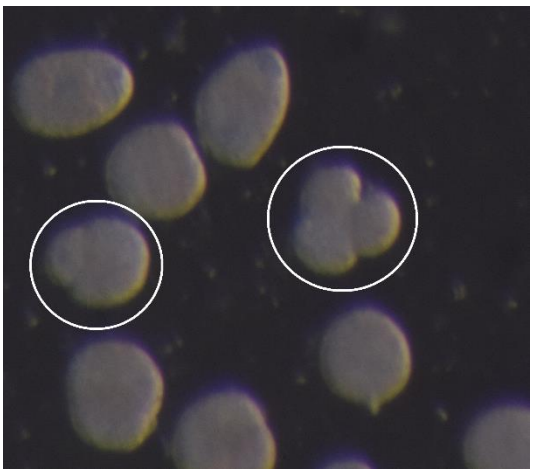


Figure 1. Crassostrea gigas oocytes after two hour incubation period. Eggs encircled in white show cleavage, denoting cell division as a result of having been fertilized, while unfertilized eggs appeared round and smooth. Three hours after fertilization commenced, fertilized eggs became nearly indistinguishable from unfertilized ones.

between samples. This aside, an improved protocol has been developed for use in further fertilization experiments.

In order to test the effect of salinity on fertilization success five salinities, at 5 PSU intervals ranging from 13 to 34 PSU, were used. Each trial lasted 10 hours, spanning 5 time intervals (Appendix 8.3). At time $t = 0$, the first fertilization period began, and it was repeated at each point in time ($t = 1, 2, 4,$ and 8). Fertilization was allowed to occur for two hours, after which samples were poured onto counting plates and fertilization success determined under a microscope (Fig 1). A sample of 200 eggs per plate were counted and the number of fertilized and non-fertilized eggs counted. To increase the number of eggs counted, three sub-samples were used, and averaged, for each sample.

To compare the effects of salinity on fertilization between treatments the proportion of fertilized eggs were analyzed with a generalized linear model with the glmer command in the lmer4 package in R 3.2.2 (R Core Team 2015). The response variable was fitted as a two-vector combination of the number of fertilized ("successes") and the numbers of non-fertilized eggs ("failures") per sample (Crawley 2013). Logit link function and a quasibinomial error structure was applied. Salinity was fitted as categorical explanatory variable, and to test for the effects of time after gamete extraction (t) on fertilization success, this was fitted as a covariate. To test for any differences in decline in fertilization success in different salinities, initial analyses also included an interaction term between salinity category and time. This was, however, dropped due to no improvement in model fit.

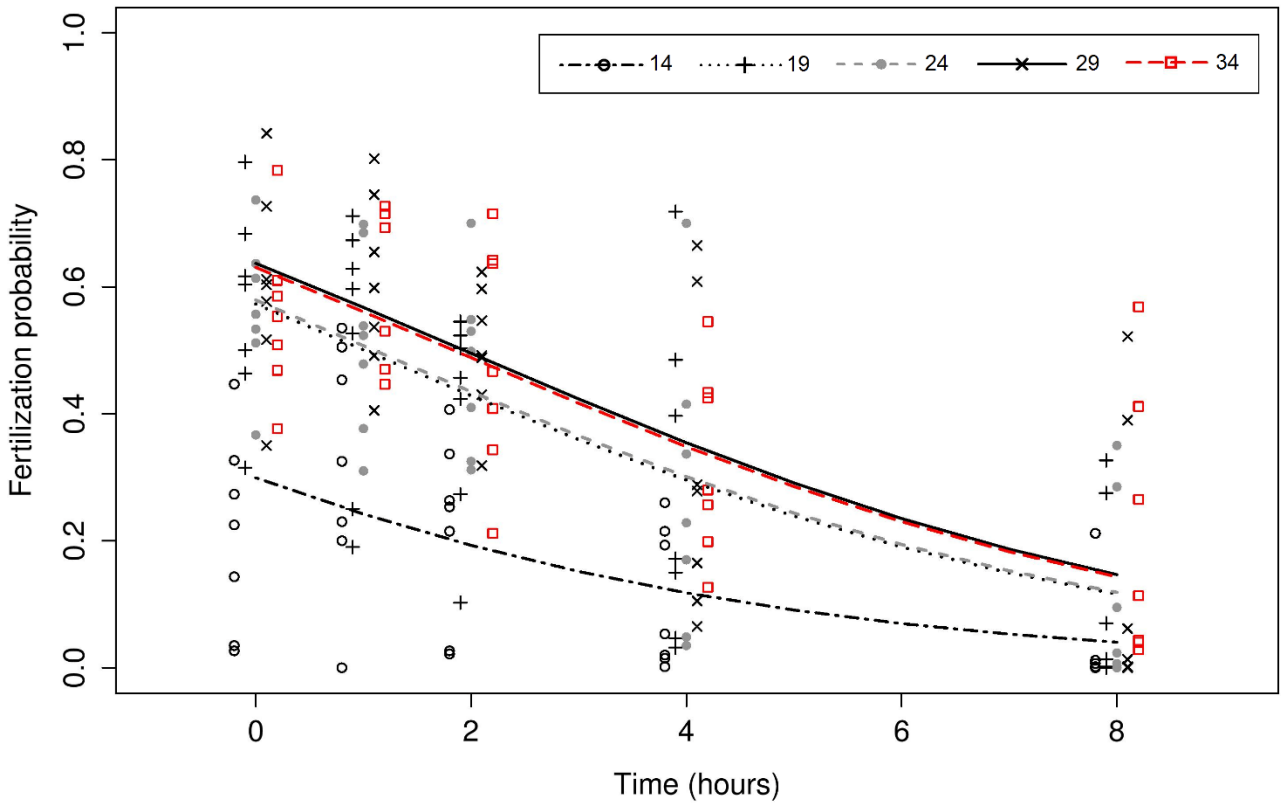


Figure 2. Fertilization probability of *Crassostrea gigas* gametes exposed to various saltwater concentrations. The legend indicates the lines -the predicted values from the logistic regression, transformed back to original scale- and the markings representing each salinity (PSU) tested. Each mark is the average fertilization proportion, for one salinity, at 5 time points for each of seven trials.

In agreement with the initial predictions, the results show an overall decrease in fertilization over time with decreasing salinity. Yet, the effect of salinity was only apparent in the lowest treatment (Fig. 2) when compared to the baseline salinity ($p<0.001$). Fertilization success declined over time, at the same rate (appendix 8.2, $p<0.001$) in all treatments. Variations within treatments, as high standard error values (Appendix 8.3) denote large variability between samples.

The effect of saltwater exposure time on gametes shows a negative trend on fertility (-0.290 $hour^{-1}$ +/- 0.028, appendix 8.3). All five treatments display the same decreasing rate of fertilization probability, as indicated by the lack of significant interaction between salinity and time. Aside from the lowest salinity (14 PSU), no differences in fertilization were seen between the baseline salinity and the remaining treatments. While differing fertilization levels are visible for individual replicates (appendix 8.4.1), high within-treatment variation (appendix 8.3) demonstrates there are noticeable differences between treatments, aside from the lowest salinity treatment.

12 Discussion

Gamete viability, represented by decreased fertilization success, of Swedish Pacific oysters was negatively affected from the exposure to the lowest salinity tested (14 PSU). Increased exposure time of spawned eggs and sperm to water had a linear and inverse proportional effect on fertilization. The decreasing fertilization rate, over time, was consistent in all treatments (Fig. 2), and salinity only had a significant effect when salinity levels dropped below the population's optimal range (Dolmer et al 2014). The results obtained are consistent with environmental tolerance of the species in the wild. In Sweden, oysters are found in waters of salinities higher than 20 PSU (Wrange et al 2009, Laugen et al 2015).

Because early organismal life stages tend to have less developed homeostatic mechanisms, making them more sensitive to disturbances, it was expected that decreases in salinity would negatively impact gamete viability. The data does show this is the case but, with the exception of the lowest salinity, the large variation of the treatments indicates a minimal salinity effect above 18 PSU. And even though the lowest salinity tested was 6-7 PSU below the species' lower salinity range in Sweden, gamete viability was significantly lower but still led to embryogenesis in most trials (Fig. 2). This is an indication that the limiting factor of *C. gigas* range expansion in Sweden may not be due to the effect of hyposaline waters on pre-embryonic stages of the species. Once fertilization occurs, the more saline-tolerant life stages could carry on their life cycles, eventually becoming established in these waters. Studies of low saltwater effects on various oyster life stages are needed in order to understand if indeed embryos are the best indicators of salinity sensitivity.

Viable *C. gigas* populations outside of Sweden are exposed to significantly lower salinities than that of the population used in this study. And while oysters throughout Europe are exposed to various stressors, such as high sea surface temperatures in the summer, Northern European populations must endure lower water temperatures and winter freezing (Wrange et al, 2009). One possible explanation for survival at high latitudes involves temperature acclimation to colder waters, potentially at the expense of tolerance to hyposaline conditions. Allocating resources in favor of mechanisms that increase temperature tolerance could limit the populations' ability to withstand decreases in salinity. Because of a lack of predation (Table 1), oysters could allot more resources for reproduction and temperature tolerance. This experiment's results suggest that since *C. gigas* is currently limited to waters with moderate to high salinity levels, >20 PSU (Wrange et al, 2009), the studied population may either not yet have developed hyposaline tolerance, or the synergistic effect

of low salinity and low water temperatures overwhelm the oysters' regulatory mechanisms and prevent a further spread of the population.

Further experiments could explore the combined effects of salinity and temperature on both gametes and early *C. gigas* developmental stages. In the near future if surface water temperatures continue to rise and salinity levels decrease, a wider expanse of habitat could potentially be occupied by the Pacific oyster. We would need to use date from climate models to set up experiments in order to subject oyster gametes and embryos to these expected conditions. And while Laugen et al (2015) have looked into *C. gigas* niche modeling, by mapping the expected habitat availability for this species, experimental data is crucial to test these predictions.

An anomaly noticeable only when looking at individual replicates (appendix 8.4), is seen in all salinities at *t = 1*. In most trials, fertilization appears to peak at this time, and not at *t = 0* as was expected. Two effects could possibly have affected this outcome. Stephano & Gould (1998) noted that polyspermy affected *C. gigas* fertilization up to 1.5 hours after oocytes were incubated in water. In this experiment, at *t = 0*, both eggs and sperm were allowed to undergo fertilization following one hour incubation period. Because fertilization occurs rapidly, within 10 minutes of gamete exposure (Song et al, 2009), polyspermy blockage might have still been present during the first time period, while at *t = 1*, two hours post-incubation, this effect would have been absent. Alternatively, one-hour incubation time may not have sufficed for complete oocyte turgidity, allowing for a maximization of gamete compatibility. Inside females, eggs are tightly packed in a hypertonic state, enabling a larger number of stored eggs within gonads. Once spawning occurs and eggs are exposed to water, they expand through osmosis and become fecund. It is possible oocytes do not reach their ideal turgid state until after one hour after being exposed to water (Havenhand, pers comm).

12.1 Errors and Limitations

The goal of this experiment was to test the effects of reduced seawater salinity on gamete viability and fertilization success, as well as the longevity of gametes after extraction. Yet, since both oocytes and sperm were subjected to the experimental treatment, it is not possible to deduce whether it was the gametes, eggs, or both, which were affected. Eggs were allowed to acclimate while sperm stocks were diluted directly in the five saltwater concentrations (Appendix 8.1). The results, though, point towards lower combined gamete viability, leading to decreased embryonic formation in hyposaline waters. Such results remain relevant in an ecological perspective, as the

species would be unable to continue expanding its range if fertilization does not occur. For this Swedish oyster population, while it is uncertain whether female or male gametes are hampered by lower salinity levels, the initial threshold of fertilization appears to be overcome, albeit by a small margin. This is seen in the non-zero fertilization success of *C. gigas* gametes exposed to waters of lower salinity than the population's optimal range.

Due to time constraints, neither pH nor oocyte concentration were standardized. While previous work by Havenhand and Shlegel (2009) shows no effects of decreased pH on sperm motility and viability, not much is yet known about its effect on eggs. Moreover, by looking only at the earliest life stage of a species we are unable to conclude that one environmental parameter, in this case, salinity, will prevent or hasten their spread. Fertilization could potentially be low, yet survivability into the more developed stages may be higher. Studies on the effects of various abiotic factors, including pH and salinity, on different *C. gigas* life stages are currently underway at Tjarnö. Their outcomes will help shed a light on the possible effects climate change will have on oyster reproduction as well as the species' distribution.

Salinity also varied minimally between trials. Because the saltwater utilized in these experiments was obtained near the laboratory, marginal salinity fluctuations, of 1 or 2 practical salinity units, were observed. Therefore, for the last three trials, the salinity ranged from 13 to 33 PSU. Yet, since wider fluctuations can be observed in littoral environments, this minor variation should not affect the overall pattern observed in the results.

13 Conclusion

The survival and rapid colonization of the Pacific oyster, *Crassostrea gigas*, in the Western coast of Sweden can be partly attributed to the population's tolerance to low salinity waters. This experiment tested the assumption that if water salinity is decreased, away from the population's optimal range, fertilization success will decline. Results indicate that waters of salinity lower than 20 PSU considerably reduce the fertilization probability of *C. gigas* gametes. Even though fertilization was greatly reduced in the lowest salinity, it was not completely impaired, as embryogenesis still occurred, albeit in low numbers. Such results are indicative of this population's tolerance to waters outside of its current range. And while salinity does decrease the proportion of eggs that are fertilized, it may be the combination of factors, especially with low winter temperatures, that limit the species' expansion.

Works Cited

Cadée, G.C., (2001). Herring gulls learn to feed on a recent invader in the Dutch Wadden Sea, the Pacific oyster *Crassostrea gigas*. *Basteria* 65, (2001), 33-42

Cardoso J, F.M.F., Langlet D, Loff J,F., Martins A.R., Witte I.J J., Santos P.T., W. van der veer H. (2006). Spatial viability in growth and reproduction of the Pacific oyster *Crassostrea gigas* (Thunberg 1793) along the west European coast. *Journal of Sea Research*, 57 (2007), 303-315

Child A.R., Laing, I., (2008). Comparative low temperature tolerance of small juvenile European, Ostrea edulis L., and Pacific oysters, *Crassostrea gigas* (Thunberg). *Aquaculture Research*, 29, 2 (2008), 103-113

Crawley, M.J., (2013). The R Book. London, Wiley. 2nd Ed, 2012, Print.

Dolmer, P., Holm, M.W., Strand Å., Lindegarth, S., Bodvin, T., Norling, P., Mortensen, S., (2014). The invasive Pacific oyster, *Crassostrea gigas*, in Scandinavian coastal waters: A risk assessment on the impact in different habitats and climate conditions. *Fisken og Havet*, 2-2014

Fabioux, C., Huvet, A., Lapegue, S., Heurtebise S., Boudry P. (2002). Past and present geographical distribution of populations of Portuguese (Crassostrea angulata) and Pacific (*C. gigas*) oysters along the European and North African Atlantic coasts. *Haliotis* 31, (2002), 33-44

Flores-Vergara, C., Cordero-Esquivel., B., Ceron-Ortiz A.N., Arredondo-Vega B.O. (2004). Combined effects of temperature and diet on growth and biochemical composition of the Pacific oyster Crassostrea gigas (Thunberg) spat. *Aquaculture Research*, 35, (12), 1131-114

Grizel, H., Heral, M., (1991). Introduction into France of the Japanese oyster (*Crassostrea gigas*). *Conseil International pour l'Exploration de la Mer*, 47, (1991), 399-403

Havenhand, J.N., Shlegel, P, (2009). Near-future levels of ocean acidification do not affect sperm motility and fertilization kinetics in the oyster *Crassostrea gigas*. *Biogeosciences*, 6 (2009), 3009–3015

Hollander, Johan, Blomfeldt, Johan, Strand, Å. (2015). Effects of the alien Pacific oyster (Crassostrea gigas) on subtidal macrozoobenthos communities. Marine Biology 162 (3): 547-555.

IPCC, 2014: Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, R.K. Pachauri and L.A. Meyer (eds.)]. IPCC, Geneva, Switzerland, 151 pp.

Knowles, G., Handlinger, J., Jones, B., Moltschaniwskyj, N. (2014). Hemolymph chemistry and histopathological changes in Pacific oysters (*Crassostrea gigas*) in response to low salinity stress. *Journal of Invertebrate Pathology* 121 (2014), 78–84

Ko, W.K.G., Chan, B.S.V., Dineshram R., Choi, K., Dennis, S., Adela, L.J., Yu Z., Thiyagarajan, V. (2013). Larval and Post-Larval Stages of Pacific oyster (*Crassostrea gigas*) Are Resistant to Elevated CO2. *Plos One*. Vol. 8, No. 5, 2013

Laugen, A.T., Hollander J., Obst, M., Strand, Å. (2015). The Pacific oyster (*Crassostrea gigas*) invasion in Scandinavian coastal waters in a changing climate: impact on local ecosystem services. Book chapter in: Canning-Clode, J (Ed.). *Biological Invasions in Aquatic and Terrestrial Systems*: Biogeography, Ecological Impacts, Predictions and Management. 2016

Miossec, L., Le Deuff, R., Goulletquer, P. (2009). Alien species alert: *Crassostrea gigas* (Pacific oyster). *ICES Cooperative Research Report*. No. 299, 2009

Nehring, S. (2006): NOBANIS – Invasive Alien Species Fact Sheet – Crassostrea gigas. *Online Database of the North European and Baltic Network on Invasive Alien Species*. https://www.nobanis.org/.../c/crassostrea-gigas/crassostrea-gigas.pdf. October 2015

R Core Team (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/. February 2016.

Stephano, J. L., Gould, M., (1988). Avoiding polyspermy in oyster (*Crassostrea gigas*). *Aquaculture*, 73, 1-4, (1988), 295-307.

Strand, Å., Waenerlund A., Lindegarth, S., (2011) High Tolerance of the Pacific oyster (*Crassostrea gigas*, Thunberg) to Low Temperatures. *Journal of Shellfish Research*, Vol. 30, No. 3, 733–735, 2011

Song, Y.P., Suquet, M., Queau, I., Lebrun, L. (2009). Setting of a procedure for experimental fertilization of Pacific oyster (Crassostrea gigas) oocytes. *Aquaculture*. Vol. 287. No. 3-4, (2009), 311-314

Troost, K. (2010), Causes and effects of a highly successful marine invasion: Case-study of the introduced Pacific oyster *Crassostrea gigas* in continental NW European estuaries. *Journal of Sea* Research 64 (2010), 145–165

Wrange, A., Valero, J., Harkestad L.S., Strand, Ø., Lindegarth, S., Christensen, H.T., Dolmer, P., Kristensen, P.S., Mortensen, S. (2009). Massive settlements of the Pacific oyster, *Crassostrea gigas*, in Scandinavia. *Biological Invasions* (2010), 12:1453–1458

15 Appendix

15.1 Protocols

Appendix 15.1 Dilution table. The experiment begins when eggs are placed in the initial FSW (filtered sea water) amounts at $t = 0$. Subsequent amounts of FFW (filtered fresh water), based on the dilution equation $C_1V_1 = C_2V_2$ – where C is the concentration of salt (PSU) and V is the volume of the container - are periodically added to minimize oocyte hyposaline shock.

Time	Hour	34 PSU	29 PSU	24 PSU	19 PSU	14 PSU
0:00		20ml FSW	17.06ml FSW	14.12ml FSW	11.176ml FSW	8.235ml FSW
0:12			2.94ml FFW	2.94ml FFW	2.94ml FFW	2.941ml FFW
0:24				2.94ml FFW	2.94ml FFW	2.941ml FFW
0:36					2.94ml FFW	2.941ml FFW
0:48						2.941ml FFW

Time	Hour	Task	Notes
t= -1		Obtain sperm (~2ml from 3 males) and eggs (1-1.5ml egg stock from each of 3 females)	
		Place egg stocks in 3 10ml Eppendorf tubes. Sperm stock in 1 3ml Eppendorf tube, store on ice	
		Refer to Salinity Protocol prior to the transfer of egg stocks.	Cut tips of yellow pipets when transferring egg stocks.
		t= -1 begins when eggs are added to Falcon tubes with FSW	
		Transfer initial amounts of FSW into each (5) 15ml F tubes for eggs	
		Add 100-150ul egg stock from each female into each of 5 15ml F tubes	Egg density per sub sample: 300-500
t= 0		Add 6ml FSW (one for each salinity) to each of 5 F tubes for sperm	Sperm stock density in each tube ~6,000-10,000 cells/ul
		Transfer sperm into each of 5 F tubes (one for each salinity) Stir.	
		Immediately after, Add 300ul sperm to t=0 egg F tubes (15 total) Stir Gently	label counting well-plates
t= 1		Add sperm to t=1 egg F tubes (15 total) Stir Gently	
t= 2		Add sperm to t=2 egg F tubes (15 total) Stir Gently	
		t=0 tubes pour onto well plates --> count 200 eggs fert/non-fert	Counting must be finished within 30 minutes after t=2
t= 3		t=1 tubes pour onto well plates --> count 200 eggs fert/non-fert	Counting must be finished within 30 minutes after t=3
t= 4		Add sperm to t=4 egg F tubes (15 total) Stir Gently	
		t=2 tubes pour onto well plates --> count 200 eggs fert/non-fert	Counting must be finished within 30 minutes after t=4
t= 5			
t= 6		t=4 tubes pour onto well plates --> count 200 eggs fert/non-fert	Counting must be finished within 30 minutes after t=6
t= 7			
t= 8		Add sperm to t=8 egg F tubes (15 total) Stir Gently	
t= 9			
t= 10		t=8 tubes pour onto well plates --> count 200 eggs fert/non-fert	Counting must be finished within 30 minutes after t=10

15.2 Fertilization protocol for the Pacific oyster (Crassostrea gigas).

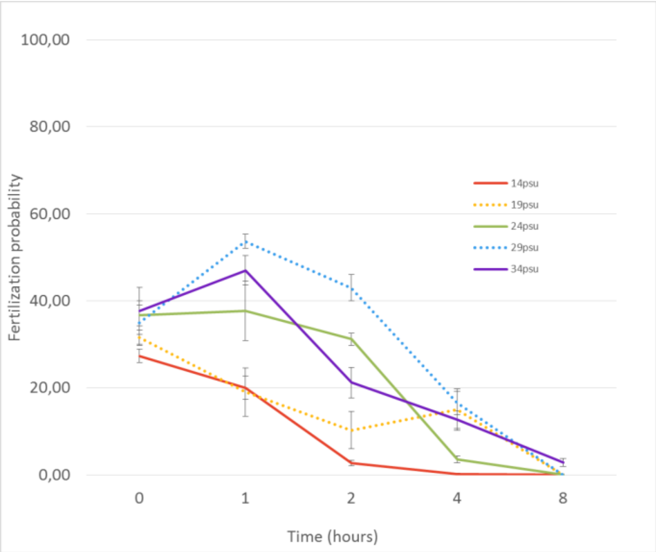
15.3 Statistical Results

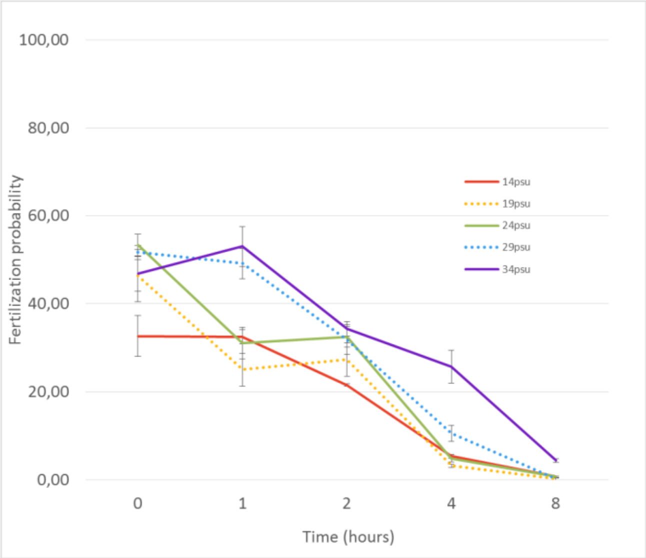
15.3 Output of the statistical results based on a generalized linear model with the glmer command in the lmer4 package in R 3.2.2 (R Core Team 2015), testing the response of fertilization success in different salinities.

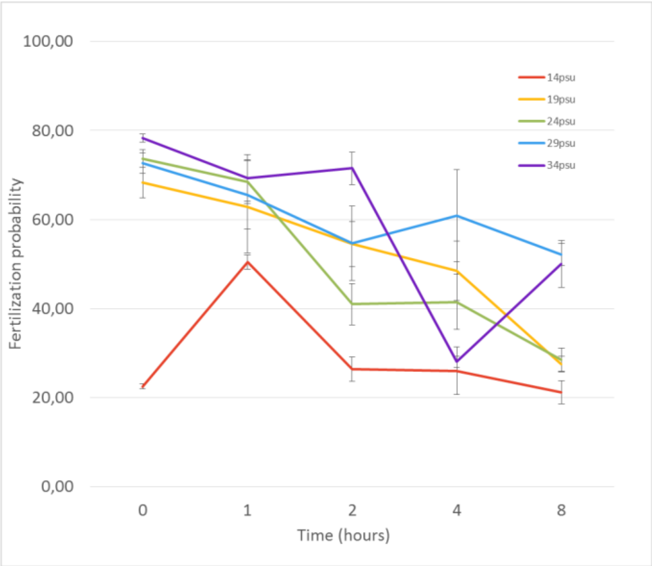
Deviance Residuals:				
Min	1Q	Median	3Q	Max
-18.237	-7.996	-1.461	6.009	24.187
Coefficients:				
	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.53441	0.15356	3.480	0.00064 ***
salcat1	-1.38537	0.22153	-6.254	3.27e-09 ***
salcat2	-0.24130	0.19843	-1.216	0.22570
salcat3	-0.21554	0.19654	-1.097	0.27438
salcat4	0.02762	0.19693	0.140	0.88865
hour	-0.29042	0.02798	-10.379	< 2e-16 ***

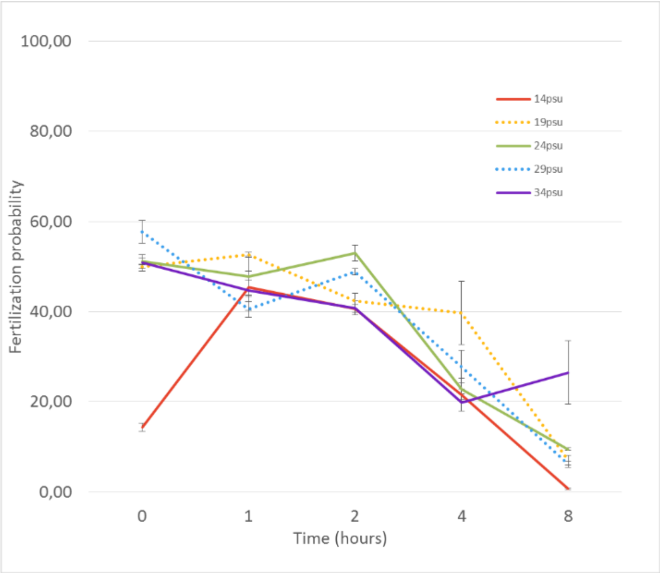
Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1				
(Dispersion parameter for quasibinomial family taken to be 85.80899)				
Null deviance: 30941 on 171 degrees of freedom				
Residual deviance: 14778 on 166 degrees of freedom				
(3 observations deleted due to missingness)				
AIC: NA				

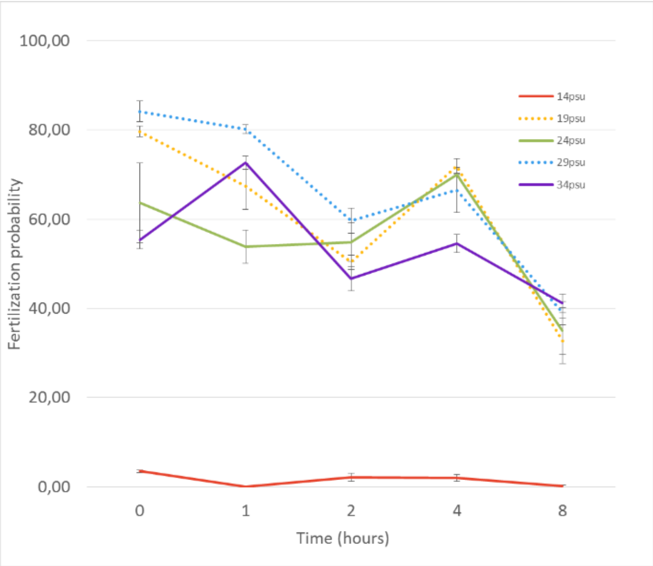
15.4 Individual Trials

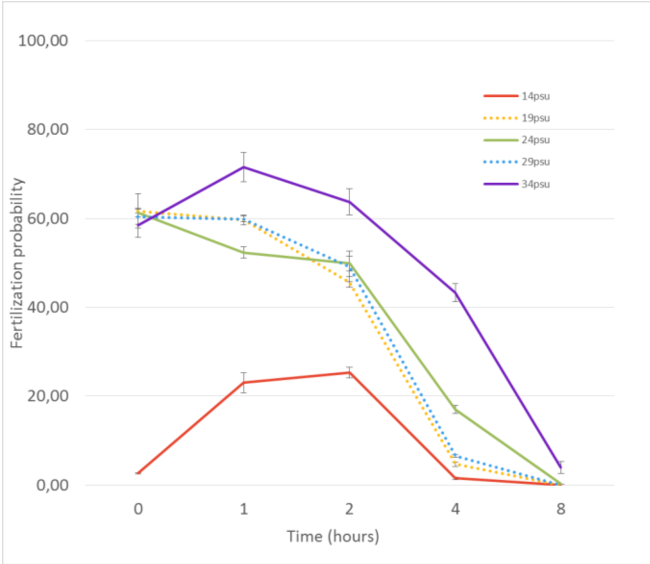


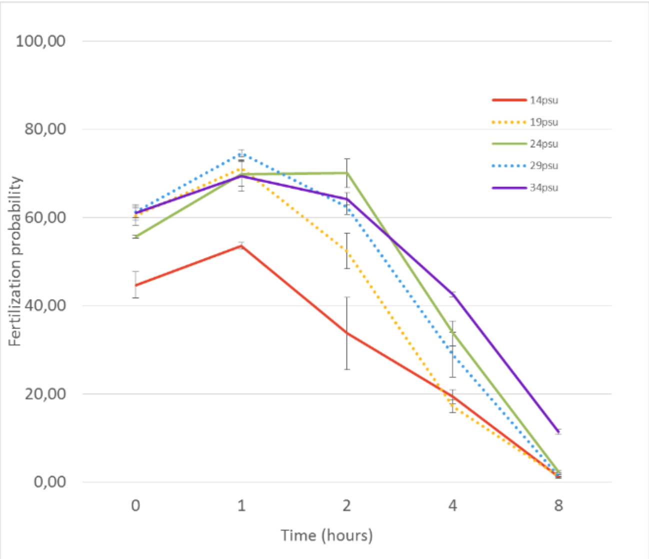












15.4. Individual trials of the fertilization experiment. Each graph represents one fertilization trial, from a triple-parental cross, over five points in time (*t = 0, 1, 2, 4, and 8),* and in 5 salinities (14, 19, 24, 29, and 34 PSU).