Examining the influence of temperature on the sex ratio of *Nereocystis luetkeana* gametophytes

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Abstract

The canopy-forming kelp, *Nereocystis luetkeana,* provides important biogenic habitat and numerous ecosystem services. There has been a documented decline of this foundation species in the Salish Sea due to increasing temperatures and nutrient limitation, among other stressors. The early, microscopic life stages of the species are particularly vulnerable to environmental stressors such as temperature. Past work has demonstrated that temperature significantly affects gametophyte growth, density, and survivorship in *Nereocystis luetkeana*, and sex-expressed genes in *Saccharina latissima*. However, the impact of temperature on the sex ratio of *Nereocystis luetkeana* gametophytes is unknown. This study explored how temperature affects the gametophyte sex ratio, the relative proportion of female to male gametophytes, of *Nereocystis luetkeana* through a temperature-controlled experiment. Zoospores were grown in treatments across their thermal tolerance (10-18°C) for 12 days, then cultures were imaged and categorically scored by their sex. We observed no difference in the mean ratio of female to male gametophytes between temperature treatments (p=0.13). However, we found that the observed gametophyte sex ratio was not the expected proportion of 0.5 to 0.5 and male gametophytes were consistently more abundant in the samples. These results suggest that warming water temperatures anticipated in response to climate change will not significantly bias the development of male and female *Nereocystis* gametophytes within their thermal tolerance. Furthermore, this study highlights the need for further research on the effects of environmental stressors on the microscopic, haploid phases of kelp to better understand population dynamics under future conditions.

Introduction

Kelp forests are hotspots for productivity and biodiversity in marine ecosystems. Dominating temperate and subpolar rocky coastlines in lower intertidal and shallow subtidal zones, they serve as both foundation species and ecosystem engineers, structuring nearshore ecosystems (Teagle et al., 2017). Kelp forests directly modify the abiotic environment by influencing nutrient cycling, seawater chemistry, light availability and wave attenuation in coastal zones (Pfister et al., 2019; Clark et al., 2004; Elsmore et al., 2022). In addition to providing key biogenic habitats and nursery grounds to numerous commercially and ecologically important marine plants and animals, kelp also supports complex food webs through primary productivity and detrital subsidies (von Biela et al., 2016; Walton et al., 2022).

An ecologically, economically, and culturally important kelp species is the bull kelp, *Nereocystis luetkeana (K.Mertens) Postels & Ruprecht 1840* (hereafter *Nereocystis*). *Nereocystis* is one of just two canopy-forming kelp species found in nearshore habitats along the west coast of North America (Scrhroeder et al., 2019). Due to its fast growth rate and canopy-forming nature it provides a complex habitat for a diverse array of species (Springer et al., 2007). *Nereocystis* ranges from the Aleutian Islands, AK, to San Luis Obispo, CA, and tends to occupy intertidal to subtidal habitats (up to 20 m deep) with high flow and unstable substrata (Druehl, 1970; Maxell & Miller, 1996). *Nereocystis* is economically valuable and creates vital habitat for many commercial fish species including abalone and rockfish (Kidder, 2006; Springer et al., 2007). Recently, *Nereocystis* has been prioritized for conservation and restoration within Puget Sound and the state of Washington at large due to its ecological importance and significant declines (Calloway et al., 2020; State of Washington, 2022).

A 2016 review indicates that global stressors such as temperature change and acidification interact synergistically with other stressors such as predation, pollution, nutrient limitation, and alterations in community structure to drive changes in kelp forest dynamics (Krumhansl et al., 2016). Work examining changes in the distribution of *Nereocystis* in the southern extremes of the Salish Sea found that kelp along wave-sheltered shorelines are more sensitive to environmental stressors, such as increased temperatures and poor nutrient conditions; researchers documented a staggering 63% loss in kelp bed extent in 2017 relative to historical baselines (Berry et al., 2023). In order to fully understand the drivers of change in kelp forests it is necessary to detangle the relative importance of specific local and global stressors, which requires resolving data gaps at spatial and temporal scales, as well as across the life cycle of kelp. Understanding how environmental conditions impact each stage of *Nereocystis'* life history is critical because early developmental stages are very sensitive to fluctuations in environmental conditions like temperature and salinity (Fredersdorf et al., 2009). Furthermore, the success of these early stages are essential to complete the species' life history.

Nereocystis is an annual species, exhibiting a heteromorphic life history with both macroscopic and microscopic stages (Figure 1). In the macroscopic sporophytic stage, the thallus consists of a holdfast, stipe, single pneumatocyst 'float', and numerous strap-like blades (Kidder, 2006). As the individual matures sorus patches develop on the blades, with more mature sori found at the distal ends; mature sori achieve a rich brown color, detach from the blade and drift to the benthos where they release zoospores during the summer and autumn. The zoospores settle on the benthos, where they may remain dormant for several months before developing into sexually dimorphic male and female gametophytes that produce sperm and egg. The haploid zoospore phase is a critical stage in development due to the dispersal and settlement that occurs (Lind & Konar, 2017). During fertilization, the male gametophyte releases sperm that settles on the egg of a female gametophyte, producing a zygote that develops directly on top of the female, eventually growing back into a *Nereocystis* sporophyte.

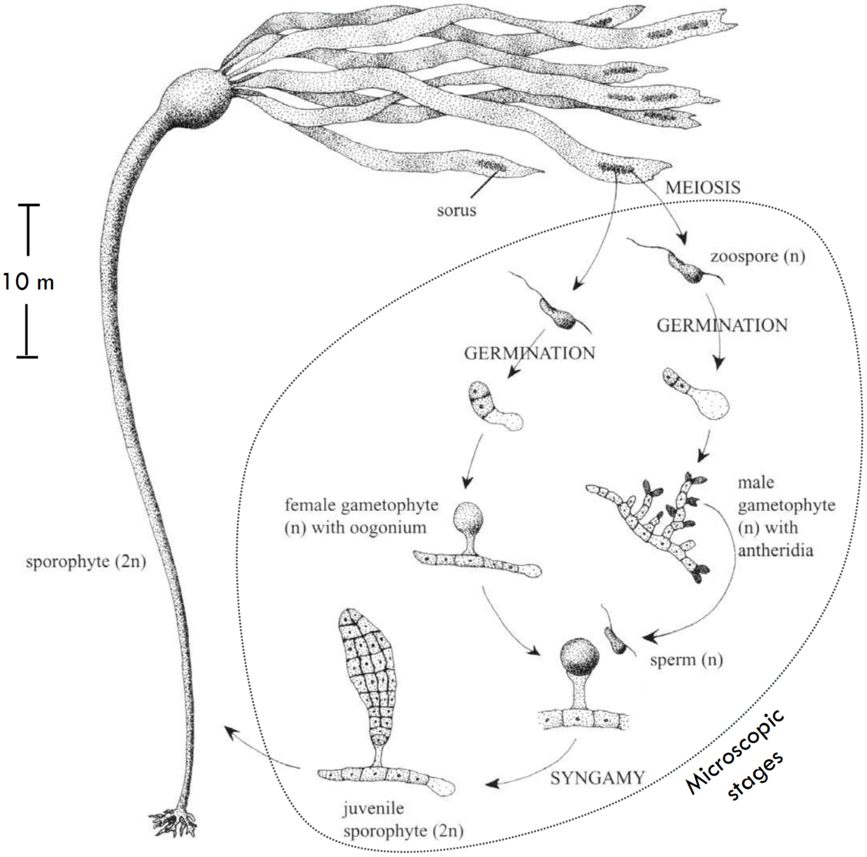


Figure 1. *Nereocystis* demonstrates an alternation of heteromorphic generations with both macroscopic and microscopic phases. Illustration by Lisa Spitler in Mondragon and Mondragon (2003).

There is still much work to be done examining survivorship across the various life history stages of *Nereocystis* under different environmental conditions. Monteiro et al. (2019) investigated the response of sex-differentiated genes within *Sacharina latissima* gametophytes when exposed to different temperatures and found that males were more resilient to increased temperature conditions, with less expression of energetically-costly regulating genes compared to the female gametophytes. Recent work by Weigel et al. (pre-print) regarding *Nereocystis'* early life stages found that temperatures exceeding 18℃ had a severe effect on sporophyte and gametophyte growth and densities, whereas nutrient availability had little effect. However, the impact that various environmental stressors have on other critical life-history aspects of *Nereocystis*, such as the sex ratio of gametophytes, remains unexplored.

The objective of this study was to determine the sex ratio of female and male *Nereocystis* gametophytes under different temperature regimes across their thermal tolerance. Based on previous research, we hypothesize that temperature influences the sex ratio of *Nereocystis* gametophytes by differentially affecting the successful development of zoospores into male and female gametophytes.

Materials and Methods

We collected fertile *Nereocystis* sori (i.e., kelp reproductive structures) at two sites on San Juan Island, Washington, and tested the effect of four temperatures (10, 13, 15, and 18℃) on the differential development of male and female gametophytes. Deadman Bay (48°30'46.7"N 123°08'46.3"W) and Friday Harbor (48°32'43.5"N 123°00'44.1"W) were selected as collection sites due to the presence and accessibility of *Nereocystis* (Figure 2). The sorus collection sites were a semi-representative selection of the distribution of kelp around the island; multiple collection sites were used to exclude any site-specific fitness patterns of the parent populations. We conducted our experiment in the summer of 2023 at Friday Harbor Laboratories.

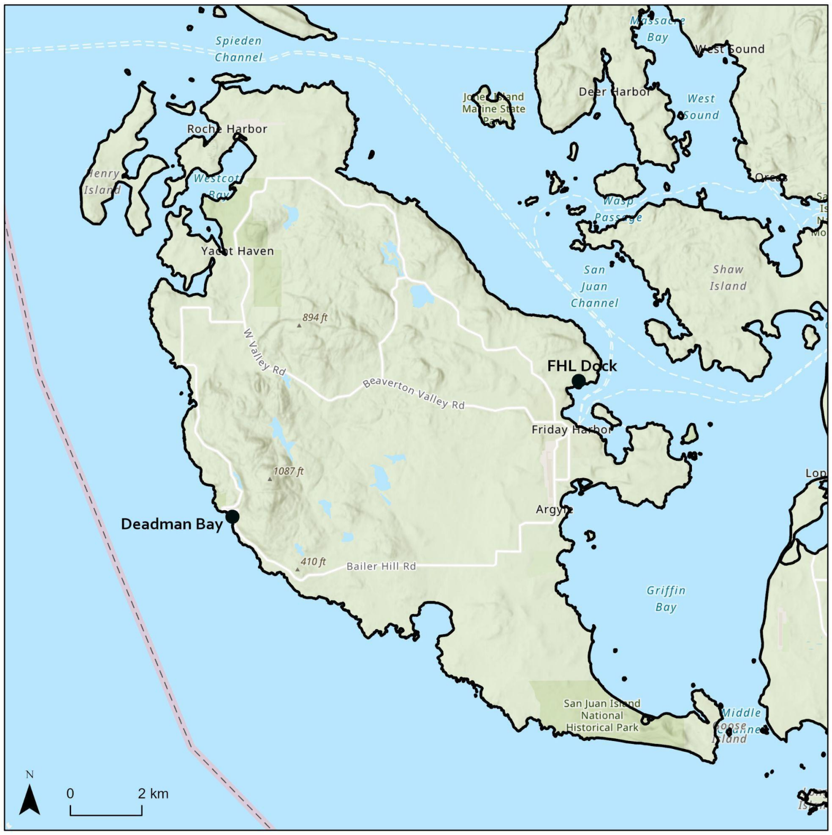


Figure 2. *Nereocystis* sori were collected from Deadman Bay and Friday Harbor Laboratories on San Juan Island, WA.

Sori collection and spore release

Blades with sori were collected from attached individuals via kayak at Deadman's Bay and opportunistically from an individual that drifted to the Friday Harbor Laboratories dock. Upon collection, we placed blades into buckets filled with ambient seawater during transport back to the lab. Approximately three to five fertile sori were collected from ten individuals at Deadman Bay and one individual at Friday Harbor. Sea surface temperature was measured at the time of collection to ensure that all sori from a given site originated from similar environmental conditions.

At the lab, we brought sori to a 10℃ temperature-controlled room. Prior to spore release, we cleaned the blades. First, we lightly scrubbed the blades with paper towels then flushed them with ambient seawater to remove any epiphytes or excess mucilage. Then, we individually dipped the blades in a dilute betadine solution (1 mL povidone-iodine 10% antiseptic solution in 400 mL pasteurized seawater (i.e., seawater heated to 80°C for 1 hour)). Finally, we thoroughly rinsed the blades in pasteurized seawater before individually placing them between layers of damp paper towels in a glass container. The container remained in the 10°C room in the dark for 18 hours to condition the sori for synchronized spore release (Deiman et al. 2012).

We removed five to seven sori and placed them within a glass beaker filled with 800 mL of pasteurized seawater. After approximately one hour at 10°C the *Nereocystis* spore solution became dark from the release of spores and mucilage.

We removed the spent sori and filtered the spore solution through four layers of cheesecloth to remove excess mucilage (Lind & Konar, 2017). We estimated the spore concentration with a hemocytometer to be 1,392,500 spores mL-1. Then we diluted the spore solution with pasteurized seawater to a final concentration of 12,500 spores mL-1 solution.

Temperature experiment

To test the effects of temperature on the sex ratio of *Nereocystis* gametophytes we conducted a temperature-controlled experiment over the first 12 days following zoospore release. Previous research conducted by Weigel et al. (pre-press) indicates that temperatures between 10-18°C are optimal for gametophyte growth and densities, and temperatures above 20°C are fatal. Therefore, we used four temperatures ranging from the ambient sea surface temperature at the time of collection (10°C) to the upper thermal tolerance (18°C).

We set up four five-gallon open-topped glass aquarium tanks filled with fresh water in a 10°C room. We submerged one Finnex Titanium 500 aquarium heater in each of the 13, 15, and 18°C treatments and regulated temperatures with Inkbird Aquarium temperature controllers to achieve the desired temperature conditions; the 10°C treatment matched the ambient temperature of the room so no additional heating or cooling devices were implemented. We monitored the temperatures of each treatment for the duration of the study using HOBO Pendant MX Water Temperature Data Loggers as well as standard alcohol lab thermometers. To ensure a constant temperature throughout the tank, we installed a Hygger aquarium air pump outfitted with plastic tubes and an air regulator to keep water circulating throughout the 13, 15, and 18°C treatments (Figure 3). We imposed temperature conditions 24 hours prior to the start of the experiment.

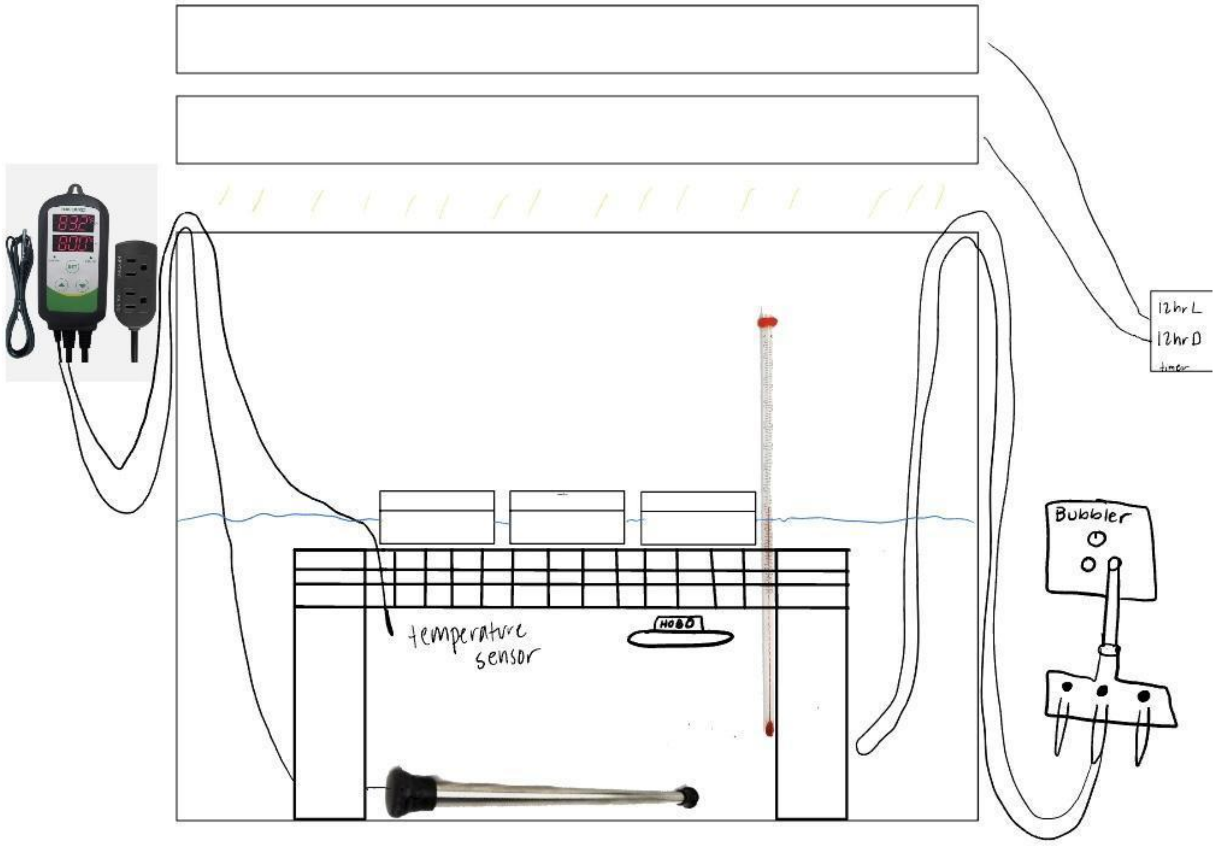


Figure 3. Experimental set-up of tanks used for temperature treatments 13 , 15 , 18°C. For the 10°C treatment, everything except the bubbler, heater, and temperature controller were present within the tank. All four treatment groups were under two 1.2m 40-watt fluorescent lights.

Cultures were kept under an irradiance level of 30-40 PAR (µmol m-2 s-1) using cool white (5000K) fluorescent lights and a mesh screen to diffuse the light. Lights were connected to timers to establish a photoperiod of 12 light hours and 12 dark hours each day to mimic springtime environmental conditions.

We used three six-welled well plates for each temperature treatment, resulting in 18 replicates per treatment. We pipetted 10 mL of our 12,500 spores mL-1 solution into each well for all four temperature treatments. After placing the solution into each well, we wrapped each well plate in Parafilm to sufficiently seal the plates to maintain constant salinity by preventing water from the temperature baths from entering and water loss from evaporation. In order to keep the well plates partially submerged in the freshwater baths, we constructed stands from PVC and covered the upper surface in plastic mesh secured with zip ties. Once well plates were sealed, we placed them directly into the corresponding treatments without acclimation, leaving them half submerged in the temperature baths.

We maintained treatment conditions by recording the temperature, and checking the performance of the pump and lights each morning and night. We also refilled the freshwater baths as needed, to keep the well plates half submerged. To ensure equal light conditions for all replicates we used R to generate random sequences of positions (see Appendix); the same sequence of well plates was used in each of the treatments on a given day.

To check the progression of zoospore development, we used a Nikon inverted microscope (Model Eclipse TE2000-U) on day four to observe one well plate from each treatment. The developing gametophytes were less mature than expected, so on day five we replaced 5 mL of the original growing medium with sterile F/2 solution (5 drops each of ProLine water conditioner A and ProLine water conditioner B into 1 L of pasteurized seawater; entire solution was repasteurized). We used Parafilm to reseal the well plates. On day nine, we checked the progress again using a Nikon inverted microscope and decided to terminate our experiment on day 12, based on gametophyte development and time constraints.

On day 12, we removed well plates one by one, starting with our 10°C treatment. We placed each well plate onto the center stage of the Nikon inverted microscope. Starting in the upper left corner well, we serially imaged each well in QCapture Suite Plus by randomly selecting three positions within the wells.

Data Analysis

We exported all images from QCapture Suite Plus and imported them into ImageJ. We analyzed the three images from each well in batches; gametophytes within each image were scored as unknown (0), female (1), male (2), or undeveloped (3) and automatically tabulated (Figure 4). Gametophytes that were at least 50% within the field of vision were scored. Clusters of gametophytes were counted as a single "unknown".

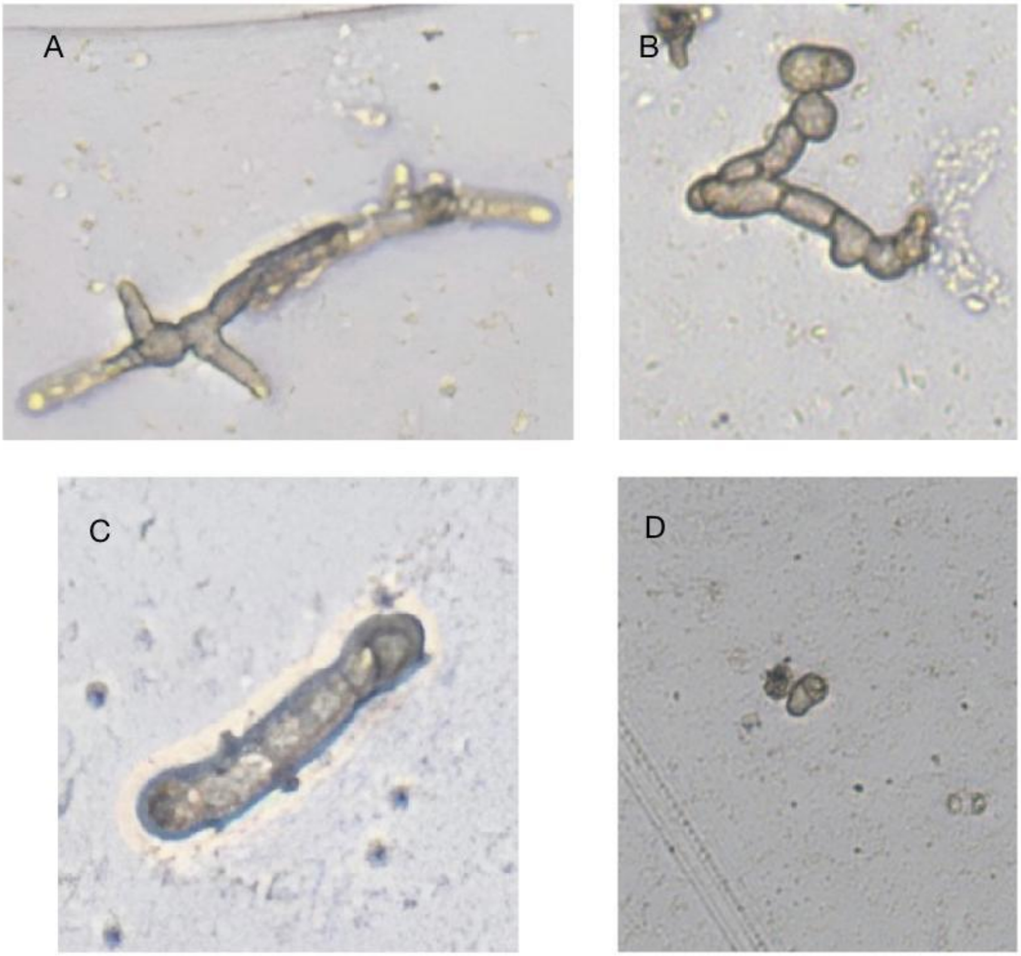


Figure 4. Representative images of A) male, B) female, C) unknown, and D) undeveloped *Nereocystis* gametophytes after 12 days of growth. Female gametophytes have noticeably thicker uniseriate filaments than their male counterparts.

We exported the automatic count data from ImageJ as comma-separated values files and imported them into a data frame in RStudio. We summed the count data for each well, resulting in 72 total data points. Using the data frame in RStudio, we separated each respective temperature treatment group, generated the ratio of female to male gametophytes in each well, and calculated the mean sex ratio within each treatment group. We then combined the female to male ratios into a single data frame so that we could run an ANOVA to compare the means of all four treatments.

We also looked at the number of undeveloped gametophytes within each temperature treatment, and used Excel to run an ANOVA to compare the average number of undeveloped gametophytes within each treatment.

To compare the observed male and female counts to the assumed expected proportion of one female to one male, we ran a chi-square goodness of fit test, with a Yates correction to account for our two-category data.

Results

Varying temperature treatments (10, 13, 15, and 18℃) did not have a significant impact on the gametophyte sex ratio (female:male) of *Nereocystis* (ANOVA, F=1.94, p=0.13; Table 1). The gametophyte sex ratio ranged from 0.38 to 2.0 and one outlier was removed from the 10℃ treatment (Figure 5.)

Table 1. Analysis of variance (ANOVA) on the effects of temperature on *Nereocystis* gametophyte sex ratio with temperature treatments of 10, 13, 15, and 18℃.

Anova: Single Factor Sex Ratio

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
10	18	19.00333	1.055741	0.791391
13	17	12.39479	0.729105	0.130259
15	18	13.37626	0.743126	0.03779
18	17	17.25526	1.015015	0.112302

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.587483	3	0.529161	1.942731	0.131247	2.743711
Within Groups	17.97707	66	0.27238			
Total	19.56456	69				

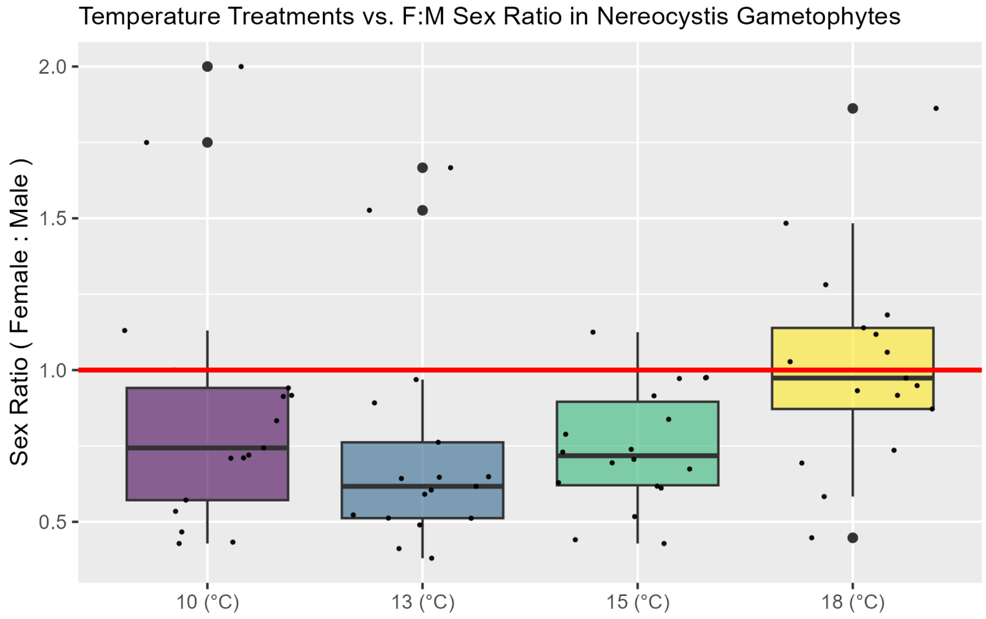
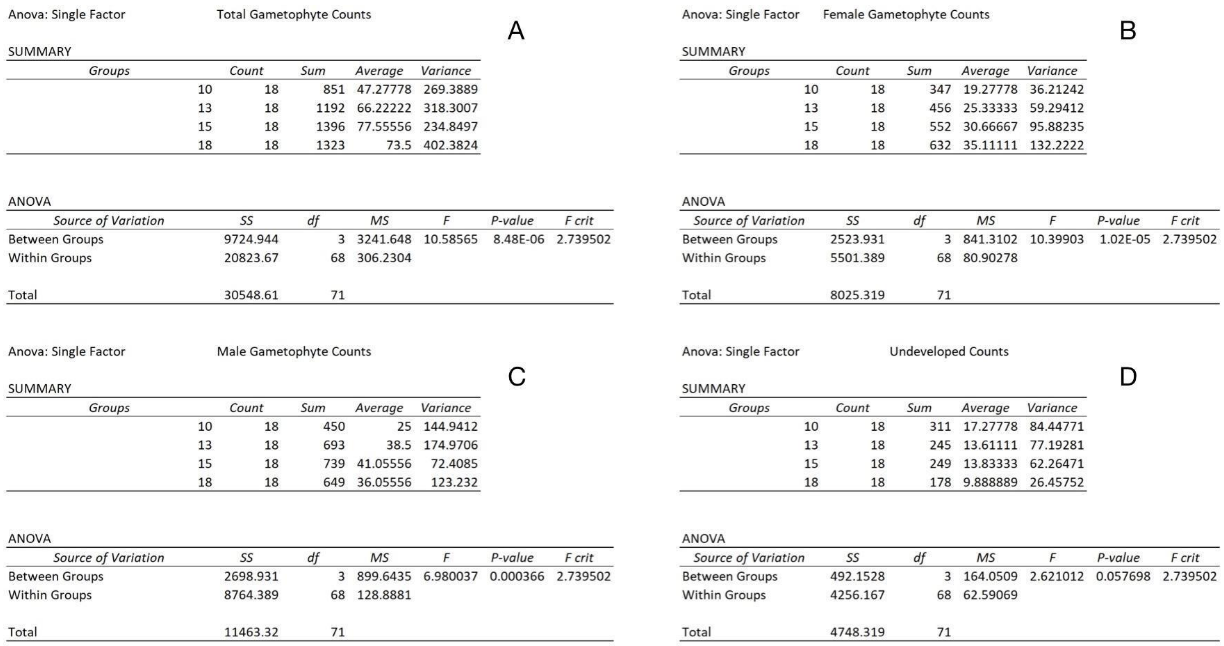

Temperature Treatments vs. F:M Sex Ratio in Nereocystis Gametophytes

Figure 5. Boxplots of *Nereocystis* gametophyte sex ratio by temperature treatment (10, 13, 15, and 18°C). The horizontal red line represents the expected ratio of female to male gametophytes.

The average abundance of undeveloped gametophytes did not differ significantly between temperature treatments (ANOVA, F=2.74, p=0.57; Table 2D), but the highest abundance of undeveloped gametophytes was in the 10℃ treatment (Figure 6). The average abundance of female, male, and total (the sum of female, male, and unknown counts) gametophytes was significantly different across temperature treatments (ANOVA, F= 2.74, p=1.02E-05; F=2.74, p=3.66E-04; F=2.74, p=8.48E-06; Table 2A-C). A post hoc Tukey test showed that the average abundance of female gametophytes was significantly different when comparing 10℃ to 15 and 18℃, and while comparing 13℃ and 18℃. For the average abundance of male gametophytes and average abundance of total gametophytes, two post hoc Tukey tests showed that there were significant differences when comparing 10℃ to 13, 15, and 18℃. We also found that there were consistently more males than females in all treatments (Figure 7).

Table 2. Analysis of variance (ANOVA) on the effects of temperature on A) the total abundance of *Nereocystis* gametophytes, B) the abundance of female *Nereocystis* gametophytes, C) the abundance of male *Nereocystis* gametophytes, and D) the abundance of undeveloped gametophytes. A-D all contained temperature treatments of 10, 13, 15, and 18℃.



A

Anova: Single Factor — Total Gametophyte Counts

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
10	18	851	47.27778	269.3889
13	18	1192	66.22222	318.3007
15	18	1396	77.55556	234.8497
18	18	1323	73.5	402.3824

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	9724.944	3	3241.648	10.58565	8.48E-06	2.739502
Within Groups	20823.67	68	306.2304			
Total	30548.61	71				

B

Anova: Single Factor — Female Gametophyte Counts

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
10	18	347	19.27778	36.21242
13	18	456	25.33333	59.29412
15	18	552	30.66667	95.88235
18	18	632	35.11111	132.2222

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2523.931	3	841.3102	10.39903	1.02E-05	2.739502
Within Groups	5501.389	68	80.90278			
Total	8025.319	71				

C

Anova: Single Factor — Male Gametophyte Counts

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
10	18	450	25	144.9412
13	18	693	38.5	174.9706
15	18	739	41.05556	72.4085
18	18	649	36.05556	123.232

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2698.931	3	899.6435	6.980037	0.000366	2.739502
Within Groups	8764.389	68	128.8881			
Total	11463.32	71				

D

Anova: Single Factor — Undeveloped Counts

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
10	18	311	17.27778	84.44771
13	18	245	13.61111	77.19281
15	18	249	13.83333	62.26471
18	18	178	9.888889	26.45752

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	492.1528	3	164.0509	2.621012	0.057698	2.739502
Within Groups	4256.167	68	62.59069			
Total	4748.319	71				

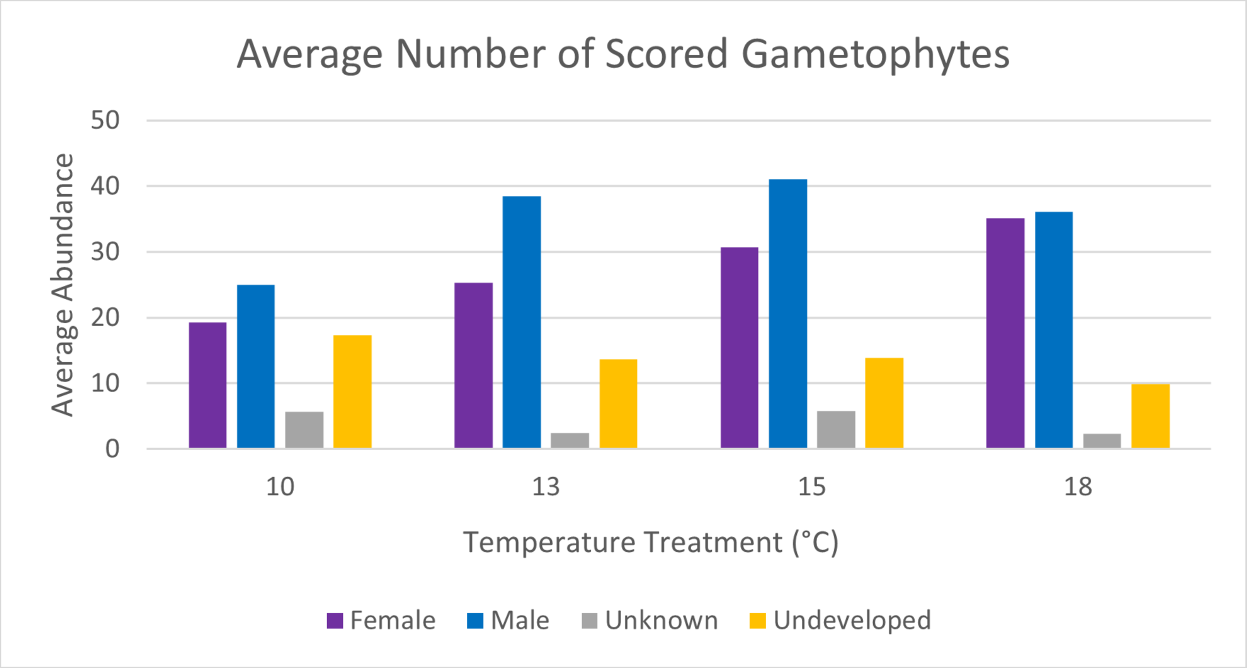


Figure 6. The effects of temperature (10, 13, 15, and 18°C) on the abundance of male, female, unknown, and undeveloped gametophytes. Average abundance refers to the total number of gametophytes in each category within a single well.

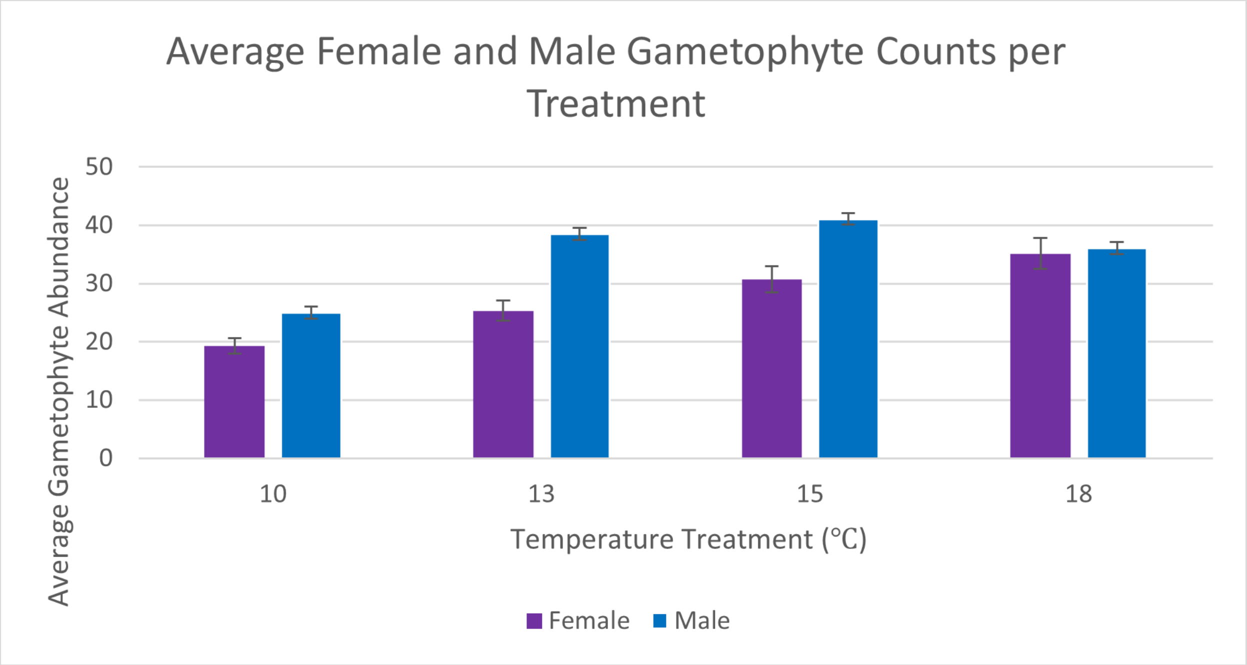


Figure 7. The effects of temperature (10, 13, 15, and 18°C) treatments on the abundance of female and male *Nereocystis* gametophytes. Abundances represent the mean ± the standard error quantity of gametophytes observed under each experimental condition.

The observed proportion of females and males was significantly different from the expected proportion of one female to one male in treatments 10, 13, and 15°C (p=2.64E-04; p=2.71E-12; p=1.95E-07) (Table 3). In the 18°C treatment, there was no significant difference from the expected proportion of one female to one male (Table 3).

Table 3. Chi-square goodness of fit test results. In the chi-square goodness of fit test, expected female:male gametophyte ratios were 50:50 across all temperature treatments (10, 13, 15, and 18°C), and a Yates correction was made to account for our two-category (male or female) data.

Treatment	x^2	df	p-value
10	13.311	1	0.000264
13	48.885	1	2.71E-12
15	27.087	1	1.95E-07
18	0.2256	1	0.6348

Discussion

Temperature has been shown to influence the early life stages of the bull kelp, *Nereocystis*, such as gametophyte growth, density, and survival (Weigel et al., pre-press). No prior research has examined the relationship between temperature and the sex ratio of *Nereocystis* gametophytes developing from zoospores. We observed in a controlled laboratory setting that changes in temperature across the thermal tolerance of *Nereocystis* did not significantly affect the gametophyte sex ratio (p=0.13). We failed to reject the null hypothesis that there were no statistically significant differences in our mean sex ratios across temperature treatments.

We found that the average abundance of female, male, and total gametophytes were significantly different across treatments. This means that although females and males occurred in roughly the same ratio across treatments, the overall abundance of gametophytes was temperature dependent, with higher temperatures (15 and 18°C) supporting a greater abundance than lower temperatures (10 and 13°C). Interestingly, the average abundance of undeveloped gametophytes did not vary significantly across treatments (p=0.57), which may suggest that higher temperatures do not alter the cycle of zoospores developing into gametophytes. However, many of these undeveloped gametophytes were larger, suggesting that they may have been developing into females, which raises questions about the required duration for the successful development of female gametophytes. If we had waited longer to score these gametophytes, these undeveloped gametophytes may have contributed to our data, which could have altered our female and male abundance, as well as the sex ratio.

During the course of our study we became interested in whether female and male gametophytes are equally likely to occur (P=0.5). Surprisingly, we found that female and male gametophytes did not occur in equal proportion at lower temperatures (10, 13, and 15°C) but were roughly equal in the 18°C condition. For treatments 10, 13, and 15°C, we rejected our null hypothesis that the observed proportions of female and male gametophytes were equally likely to occur (P=0.5), due to our significant p-values obtained from our chi-square goodness of fit test (p=2.64E-04; p=2.71E-12; p=1.95E-07). These findings suggest that we cannot assume that the proportion of female and male gametophytes that develop from released zoospores is 0.5:0.5, which raises questions about the number of available female and male gametophytes that can aid in producing gametes to then be fertilized.

Producing excess male gametophytes could possibly be a life history strategy of *Nereocystis*, as we saw there were more male gametophytes than females in all of our treatment groups, and our female and male proportions were not 0.5:0.5. Excess male gametophytes might be produced to ensure that female gametophytes will be fertilized. Male gametophytes are smaller and less energy costly than female gametophytes (Monteiro et al. 2019), which might explain why *Nereocystis* would produce more male gametophytes. More research is required to determine if this is a true life history strategy that *Nereocystis* employs. Multiple sori from various individuals would need to be collected and then monitored once zoospores are released to count the number of female and male gametophytes to understand if an excess of males are truly produced. A longer developmental period would also be necessary to ensure full development of the gametophytes into their differentiated female and male structures, which would allow for more accurate scoring.

The results of our study are limited in their applicability by several factors in our sampling protocol. Firstly, due to logistical constraints, our sample was limited to two readily accessible populations on just one island in the San Juan archipelago. Secondly, at the time of the study we had difficulty locating very ripe individuals that were obviously ready to release zoospores. These two factors contributed to our third limitation, which was that we had a relatively small sample size of around 10 individuals from Deadman Bay and just one individual harvested opportunistically from Friday Harbor. The sori from the individual that drifted to the Friday Harbor Laboratories dock were very ripe, and partially released zoospores during the incubation period so our results may represent limited genetic diversity.

Our results may also be restricted by the relatively limited development of gametophytes at the time of data collection due to culturing media and time constraints. Initially, we cultured zoospores in pasteurized seawater to mimic environmental conditions, however, we observed slow growth rates amongst the developing gametophytes and supplemented with F/2 solution part way through the experiment in hopes of enhancing the growth rate. Furthermore, logistical constraints required that we image our cultures at day 12 when gametophytes were still relatively immature. While some gametophytes were well developed and differentiated, a substantial amount were still undeveloped or not yet differentiated.

If we were to extend our research we would make changes to our collection protocol, culture conditions, and study duration. Regarding our collection protocol, we would cover a broader geographic region and select several representative sites. We would also time the start of our study to the cyclical ripening of *Nereocystis* sori as much as possible to ensure successful zoospore release from all samples. Regarding our culture conditions, we found that pasteurized seawater was an inadequate medium for growth and in future work would culture developing zoospores in nutrient-supplemented media such as F/2 from the beginning of the study. Additionally, we would reduce the concentration of zoospores from 12,500 spores mL-1 solution to around 4,000 spores mL-1 solution to reduce clumping and optimize the rate of development. Finally, under ideal circumstances, we would image the well plates every other day from day 10 onward to track the development and survivorship of gametophytes, and identify the optimum growing period for clear differentiation between females and males.

Nereocystis is a foundation species, providing essential habitat and primary productivity, as well as ecological, cultural, and commercial value to the Salish Sea. Environmental stressors that impact key taxa, such as habitat-forming subtidal kelp, may result in cascading effects throughout the community due to the complexity of ecological interactions (Scheil et al., 2004). The loss of local populations within the Salish Sea will reduce the genetic diversity of the species, as well as jeopardize ecologically and commercially important fisheries and functions that are reliant on healthy kelp beds. Therefore, it is critical to understand how changing environmental conditions impact this critical species across its life history. Our results indicate that with changing water temperature conditions in the Salish Sea, we may observe an impact on the overall abundance of *Nereocystis* gametophytes, though the sex ratio is not anticipated to change. The lack of effect on gametophyte sex-ratio at water temperatures between 10-18°C may be an encouraging finding for recruitment prospects in areas like the lower reaches of the Salish Sea, where observed temperature increases have been greatest (Berry et al., 2023). Nevertheless, further research is required to verify this finding through broader sampling in combination with environmental monitoring and genetic diversity testing, as well as explore the impacts of the multiple stressors kelp forests experience *in situ* on gametophyte sex ratio and other life history aspects.

Acknowledgments and Attribution

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Appendix

All data, code, and output can be viewed and downloaded from https://github.com/ecologytate/nereosisters.git.

Additionally, raw and annotated images can be viewed at https://drive.google.com/drive/folders/1g7whmSRw4iT_8c3RPEr-phlVtkkNlL4S?usp=drive_link.

Table A-1. Total gametophyte count data for each sample (n=72). 'temp' refers to the treatment condition, 'treat_well' corresponds to the specific replicate within a treatment; 'female' refers to female gametophytes, 'male' refers to male gametophytes, 'unk' refers to unknown gametophytes, and 'und' refers to undeveloped gametophytes.

Gametophyte Count Data

This table displays the temperature treatment and summed count data of three measurements for each sample.

...1	temp	treat_well	female	male	unk	und
1	T10	Counts_T10_W01	12	28	6	33
2	T10	Counts_T10_W02	13	30	11	20
3	T10	Counts_T10_W03	11	12	1	38
4	T10	Counts_T10_W04	22	31	3	27
5	T10	Counts_T10_W05	23	43	2	14

6	T10	Counts_T10_W06	29	39	3	20
7	T10	Counts_T10_W07	16	28	1	9
8	T10	Counts_T10_W08	16	17	2	9
9	T10	Counts_T10_W09	18	25	2	10
10	T10	Counts_T10_W10	21	45	0	1
11	T10	Counts_T10_W11	27	38	3	8
12	T10	Counts_T10_W12	21	5	1	22
13	T10	Counts_T10_W13	20	24	2	21
14	T10	Counts_T10_W14	21	12	4	20
15	T10	Counts_T10_W15	24	24	3	12
16	T10	Counts_T10_W16	21	23	3	15
17	T10	Counts_T10_W17	6	3	1	13
18	T10	Counts_T10_W18	26	23	6	19
19	T13	Counts_T13_W01	32	42	7	7
20	T13	Counts_T13_W02	35	21	7	3

21	T13	Counts_T13_W03	23	44	1	28
22	T13	Counts_T13_W04	21	41	4	10
23	T13	Counts_T13_W05	24	37	3	10
24	T13	Counts_T13_W06	26	44	2	26
25	T13	Counts_T13_W07	29	19	0	8
26	T13	Counts_T13_W08	22	43	7	12
27	T13	Counts_T13_W09	29	47	1	9
28	T13	Counts_T13_W10	33	37	2	20
29	T13	Counts_T13_W11	31	32	0	1
30	T13	Counts_T13_W12	26	43	3	13
31	T13	Counts_T13_W13	1	0	1	0
32	T13	Counts_T13_W14	19	50	3	29
33	T13	Counts_T13_W15	27	42	1	12
34	T13	Counts_T13_W16	33	51	0	20
35	T13	Counts_T13_W17	21	51	0	19

36	T13	Counts_T13_W18	24	49	1	18
37	T15	Counts_T15_W01	24	34	12	27
38	T15	Counts_T15_W02	15	29	5	19
39	T15	Counts_T15_W03	15	34	7	16
40	T15	Counts_T15_W04	25	36	18	22
41	T15	Counts_T15_W05	21	34	15	29
42	T15	Counts_T15_W06	15	35	5	25
43	T15	Counts_T15_W07	31	37	5	10
44	T15	Counts_T15_W08	31	46	11	10
45	T15	Counts_T15_W09	39	62	6	9
46	T15	Counts_T15_W10	45	40	6	3
47	T15	Counts_T15_W11	35	36	4	9
48	T15	Counts_T15_W12	43	47	4	9
49	T15	Counts_T15_W13	27	37	0	18
50	T15	Counts_T15_W14	40	41	3	5

51	T15	Counts_T15_W15	34	46	0	9
52	T15	Counts_T15_W16	38	39	1	8
53	T15	Counts_T15_W17	33	54	2	15
54	T15	Counts_T15_W18	41	52	1	6
55	T18	Counts_T18_W01	17	38	2	13
56	T18	Counts_T18_W02	37	38	2	9
57	T18	Counts_T18_W03	38	34	1	18
58	T18	Counts_T18_W04	34	39	4	11
59	T18	Counts_T18_W05	33	36	5	15
60	T18	Counts_T18_W06	54	29	1	14
61	T18	Counts_T18_W07	0	0	0	0
62	T18	Counts_T18_W08	37	36	2	19
63	T18	Counts_T18_W09	46	31	8	12
64	T18	Counts_T18_W10	39	53	3	13
65	T18	Counts_T18_W11	36	34	2	7

66	T18	Counts_T18_W12	34	49	1	5
67	T18	Counts_T18_W13	28	48	1	2
68	T18	Counts_T18_W14	41	36	4	9
69	T18	Counts_T18_W15	37	39	1	8
70	T18	Counts_T18_W16	39	33	1	9
71	T18	Counts_T18_W17	41	44	4	10
72	T18	Counts_T18_W18	41	32	0	4