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UNIVERSITY OF SAN DIEGO

San Diego

The effect of marine snow particle distribution on the foraging behavior of

Calanus pacificus

by

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DEDICATION

To papa, for always encouraging me to follow my dreams.

To mama, for teaching me to live life the *wabi sabi* way.

And to Sarah, for being my rock.

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ABSTRACT

Marine snow is a major component of the biological pump, through which carbon is exported to the deep ocean. The sinking of marine snow can be disrupted by organisms, including many species of zooplankton that ingest or break up aggregates. These processes can have important impacts on planktonic food web dynamics and carbon export. Marine snow can have vertically patchy distributions, occurring in thin layers, which may further affect interactions with zooplankton. In this lab-based study, we examined how the presence of a marine snow layer affects copepod behavior and ingestion.

We conducted a series of experiments in which copepods of the species *Calanus pacificus* were exposed to four different feeding environments: a layer of marine snow, a homogenous distribution of marine snow, and two control treatments without marine snow – one with a density gradient and one without a density gradient. Copepod behavior was recorded with two cameras that were set up perpendicular to one another, imaging neighboring sides of the tank. We were able to reconstruct 2D and 3D copepod tracks, allowing us to compare copepod vertical distributions and calculate copepod swimming velocity, jump frequency, and path linearity. Copepod gut fluorescence was measured after the experiments to determine differences in ingestion between treatments. Gut content analysis showed that copepods did ingest marine snow when exposed to the layer and homogenous distributions of aggregates, with potentially higher ingestion seen in the layer treatment. Behavioral analyses show significantly higher residence time of copepods in the middle of the tanks (where the marine snow layer and density

gradient were located) in the layer treatment and control with gradient treatment, with substantially higher jump frequency and substantially lower vertical velocity also seen in this region for those two treatments. These findings suggest that marine snow layers may represent regions of enhanced zooplankton foraging, providing insight into how these interactions can influence particle flux.

CHAPTER 1: Introduction

Marine phytoplankton are photosynthetic organisms that drift with the ocean's currents. As primary producers, they sit at the base of the food chain and, as such, their distribution and abundance in the water column determines the spatial and temporal distribution of marine life. In order to perform photosynthesis, phytoplankton require sunlight and thus are only found in the euphotic zone – the upper region of the ocean that reaches to a maximum depth of 200 meters – since less than 1% of light penetrates past this depth. Thus, the attenuation of sunlight with depth is important in controlling vertical distribution of phytoplankton and explains why the euphotic zone contains the vast majority of life in the ocean. Spatial heterogeneity in phytoplankton populations is also driven by nutrients, the main limiting factor of primary production in the surface ocean that can determine the abundance and composition of phytoplankton communities (Tilham 1982). This leads to the temporal heterogeneity of productivity in the ocean, in that a large influx of nutrients will result in a spike in phytoplankton concentration and nutrient depletion will limit population growth (Diehl 2002).

The primary consumers of phytoplankton are a group of organisms known as zooplankton. Among these are copepods – a group of planktonic crustaceans that are considered to be the largest group of metazoans in the ocean and, as grazers, play a significant role in mediating phytoplankton populations (Boxshall and Halsey 2004).

Aside from determining the distribution and amount of life in the oceans, phytoplankton provide biogeochemical services by impacting the network of elemental fluxes in the ocean, most significant of which is the flux of carbon within the ocean (Falkowski *et al.* 2003). While autotrophic picoplankton (<2 μm in diameter) dominate primary productivity, their small sizes and thus slow sinking speeds result in them contributing relatively little to carbon export (Michaels and Silver 1988). On the other hand, larger rapidly sinking phytoplankton, such as diatoms, are considered to control carbon flux from the surface layers of the ocean (Michaels and Silver 1988). The sinking of phytoplankton out of the surface layers into the deep ocean is, however, a complex process since the sinking of these carbon-rich particles is interrupted by interactions with animals and vertical mixing (Turner 2015). Together, these processes make up the biological pump, which is controlled by a multitude of variables.

It is estimated that the biological pump exports over 10 billion tons of carbon from the surface layers to the deep ocean a year, but only about 10% of this exported carbon reaches the bottom of the mesopelagic zone (Turner 2015), and, as such, this pump is relatively inefficient. However, this export of carbon is very important because once the carbon is in the deep ocean it can be trapped below the surface layers of the ocean for 1000 years or more (Drijfhout *et al.* 1996). When carbon is sequestered into the deep ocean, more carbon can be absorbed by the surface ocean from the atmosphere, and thus the biological pump plays an important role in mediating climate change (Turner 2015).

The efficiency of the biological pump is determined by the rate of carbon export to the deep ocean, which in turn is regulated by the sinking speed of particulate organic carbon. Most of the particulate organic carbon suspended in the water is in the form of very small particles (Eppley and Peterson 1979). This particulate matter – which consists of phytoplankton, bacteria, detritus, fecal pellets, and protozoans – has been found to aggregate into larger flocs (0.5 mm or larger in diameter) known as marine snow (Alldredge and Gotschalk 1990). While marine snow aggregates are generally less abundant than individual phytoplankton cells in the water column (Alldredge and Gotschalk 1990), these larger, rapidly sinking aggregates are primarily responsible for the downward flux of organic carbon (Fowler and Knauer 1986).

The foraging by organisms on marine snow can affect the properties of these aggregates (Jackson 1990, Jackson and Burd 1998). Karl *et al.* (1988) was the first to propose that marine snow aggregates may be fragmented as they sink through the water column. Fragmentation leads to changes in aggregate size and density (Dilling and Alldredge 2000, De La Rocha and Passow 2007), which has been found to decrease sinking speeds and reduce their downward flux (Alldredge and Gotschalk 1988, Goldthwait *et al.* 2004, Prairie *et al.* 2019, Briggs *et al.* 2020). Zooplankton play a substantial role in regulating and interrupting the downward export of organic carbon (Legendre and Rivkin 2002) through interactions with these carbon-rich sinking particles (Karl *et al.* 1988, Dilling and Alldredge 2000, Goldthwait *et al.* 2004, De La Rocha and Passow 2007). The aggregation of marine snow is primarily a physical process, where formation of

these aggregates largely depends on the concentration of particles in the water column, as well as their collision rates and stickiness (Jackson 1995). However, zooplankton can also impact the generation of marine snow by providing the materials that make up these aggregates, such as fecal pellets, exuviae, and feeding structures (e.g., larvacean houses) (Dilling and Alldredge 2000). While zooplankton can sometimes facilitate the aggregation of marine snow, they can also play a significant part in the disaggregation of marine snow flocs.

Fragmentation of marine snow – resulting in smaller and slower sinking particles – can happen as zooplankton swim and migrate through the water column (Dilling and Alldredge 2000, Goldthwait *et al.* 2004) or when zooplankton directly feed on these aggregates (Christian Briseño-Avena, pers. comm.). On the other hand, foraging on marine snow by zooplankton leads to a repackaging of organic carbon into fecal pellets (Shanks and Edmondson 1989), which sink at faster rates than marine snow flocs (Bruland and Silver 1981).

Marine snow aggregates are found in patchy distributions in the water column. Specifically, these aggregates can form thin layers at density discontinuities, typically driven by sharp changes in temperature (McManus *et al.* 2003). Given these ephemeral and heterogeneous spatial distributions, the ability of copepods to chemically detect and locate marine snow (Kiørboe 2013) may allow them to exploit these aggregates as a food source. Kiørboe (2001) showed that, as marine snow aggregates sink through the water column, bacteria hydrolyze particulate organic matter and inorganic nutrients, leaving behind a plume of enhanced solute concentration. Kiørboe and Thygesen (2001) suggest

that these plumes make chemosensory detection of sinking aggregates by copepods possible. Other studies on chemodetection have shown that copepods exhibit a very distinct swimming behavior when following a chemical trail, such as one left behind a mate (e.g., Yen *et al.* 1998); this swimming pattern has been termed casting behavior, in which the copepod casts with equal frequency in all directions to maximize the probability of encountering the trail, resulting in a spiral pattern (Weissburg *et al.* 1998). Lombard *et al.* (2013) observed live copepods exhibiting casting behavior upon encountering an artificial chemical plume, with similar properties to the plume of a sinking marine snow aggregate, further confirming the role of chemodetection in the ingestion of marine snow by copepods.

The feeding by mesozooplankton on marine snow aggregates also has implications for energy transfer in the pelagic food web. For example, since copepod grazing is strongly affected by particle size (Frost 1972), the aggregation of cells too small to be consumed by copepods into marine snow aggregates may allow for a short-cut in the food chain (Lampitt *et al.* 1993). Marine snow houses a rich community of protozoans and microbes (Alldredge and Silver 1988), which are not efficiently captured by mesozooplankton when they are freely suspended in the water column (Alldredge 1972, Lampitt *et al.* 1993). Thus, zooplankton feeding on individual phytoplankton cells versus aggregated phytoplankton in the form of marine snow may impact not only the amount of food consumed but also the composition of their diet.

Small changes in the fine-scale trophic interactions between different organisms and their environment can impact global-scale processes (Levin 1992). The ocean is more complex and dynamic than most terrestrial ecosystems in its scales and processes (Maxwell *et al.* 2015). Thus, marine ecosystems are highly susceptible to small-scale variability within the water column. Such variability often results in spatial heterogeneity in the distribution of marine organisms. Patchy plankton distributions, for example, can affect grazing rates and trophic interactions on the global scale (Prairie *et al.* 2012). In order to estimate the true impact of zooplankton interactions with sinking particles on the rate of carbon export, it is crucial to determine whether these grazers are able to alter their behavior in order to locate and feed in patches of marine snow.

In this thesis, I describe an experimental study in which we observed the foraging behavior of a species of copepod in two different distributions of marine snow: a marine snow layer and a homogenous distribution of marine snow. In doing so, we addressed the following questions:

1. Does the rate of ingestion of marine snow aggregates by *Calanus pacificus* differ between the layer and homogenous marine snow distributions?
2. Does the vertical distribution of *Calanus pacificus* differ when exposed to marine snow aggregates in a layer distribution versus a homogenous distribution?
3. Is there a difference in the swimming behavioral properties (velocity, path linearity, jump frequency) of *Calanus pacificus* based on aggregate distribution?

4. How do differences in copepod vertical distribution and behavior relate to the size and sinking velocities of marine snow aggregates?

For question one, we hypothesized that copepods would show a higher rate of ingestion, as measured by gut fluorescence, when exposed to the layer distribution than when exposed to the homogenous distribution. This was based on observations by Möller *et al.* (2002), in which high-resolution images revealed that a peak in zooplankton concentration was co-located with a peak in particle (marine snow) concentration, suggesting that this layer of marine snow aggregates could be a hotspot for zooplankton foraging.

To address questions two and three, we refer to a study by Tiselius (1992), in which copepods were exposed to a thin layer of phytoplankton and their behavior was observed. Here, the copepods exhibited higher jump frequency and higher swimming velocity to locate the thin layer of phytoplankton. Once the copepods were in the layer, they reduced their swimming speed and exhibited more horizontal swimming trajectories. Based on these observations, we predicted that copepods would display a lower vertical velocity in the layer region of the layer treatment than the homogenous treatment, and that residence time would be higher in the layer region of the layer treatment than the homogenous treatment. We also hypothesized that jump frequency would be higher in the layer treatment than in the homogenous treatment since the copepods might use this behavior to locate the layer of aggregates as Tiselius (1992) observed when the copepods were trying to locate the layer of phytoplankton. Additionally, in the experimental control treatments which contained only a sharp change in salinity (halocline)

without a phytoplankton thin layer present, Tiselius (1992) observed that copepods frequently made loops and temporarily increased their velocities when exposed to the halocline, as if searching for the presence of food. Given this, we predicted that copepod velocity would increase and path linearity – a measure of how tortuous a copepod's path is – would decrease when copepods were in the middle of the tank in the control with gradient treatment.

Finally, for question four, we hypothesized that slower sinking aggregates would be more easily tracked by copepods than faster sinking aggregates. This is based on a study by Kiørboe and Thygesen (2001), which modelled that denser aggregates sink faster and thus leave behind a more concentrated, but very long and thin plume. This decreases the chance that copepods may encounter the plume and also makes it more difficult to track (Kiørboe and Thygesen 2001). Ability to track these aggregates will be demonstrated by a specific behavior termed casting behavior, during which copepods perform high velocity turns as they approach the sinking aggregate, as was observed in Lombard *et al.* (2013). As such, we would expect to see more casting behavior, identified by a lower path linearity, when copepods are exposed to less dense, slower sinking marine snow aggregates.

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CHAPTER 2: The effect of marine snow particle distribution on the foraging behavior of *Calanus pacificus*

2.1 Introduction

The planktonic food web plays an important role in regulating both the exchange of carbon dioxide between the lower atmosphere and the upper ocean and the downward export of organic carbon (Legendre and Rivkin 2002), thus playing a large part in the biological pump. In addition, plankton foraging patterns have a significant impact on how much energy is passed up the food chain (van Someren Gréve *et al.* 2019), supporting animals in higher trophic levels including important commercial fisheries species (Mackas and Beaugrand 2010). As such, investigating the variability in the fine-scale interactions in plankton ecology is important for better estimating carbon transport (Martin *et al.* 1987), trophic energy transfer, and distribution patterns of living organisms in the coastal oceans (Pinel-Alloul 1995, Jaffe *et al.* 1998). One fine-scale interaction that has received increased attention is the ingestion of carbon-rich aggregates by zooplankton, since this trophic interaction has the potential to substantially affect carbon export despite not often being considered in models of plankton ecosystem processes (e.g., Turner 2015).

Marine snow, defined as aggregates of particulate matter (consisting of phytoplankton, bacteria, detritus, fecal pellets, and protozoans) with diameter 0.5 mm or larger, are generally less abundant than individual phytoplankton cells in the water column; however, these larger, rapidly sinking aggregates are primarily responsible for the downward flux of organic carbon (Trudnowska *et al.* 2021).

Thus, marine snow may represent a disproportionately important vehicle for carbon export to the deep ocean. Although marine snow aggregates are ubiquitous throughout the world's oceans (Gorsky *et al.* 2000), field observations show that distributions vary both temporally and spatially (horizontally and vertically) throughout the water column (McManus *et al.* 2003). Several field studies have reported observing accumulations of marine snow aggregates at density discontinuities in the water column, resulting in thin layers up to 3.5 m thick, horizontally extending over kilometers, and persisting for days (McIntyre *et al.* 1995, Alldredge *et al.* 2002, McManus *et al.* 2003, Prairie *et al.* 2010). Prairie and White (2017) demonstrated that the formation of these thin layers can result from a decrease in the sinking velocity of aggregates at density interfaces, defined as delayed settling behavior.

In addition to aggregates playing a role in carbon export, they also may represent an important food source for zooplankton. Multiple field studies have observed zooplankton directly attached to marine snow aggregates (Steinberg *et al.* 1994, Green and Dagg 1997, Shanks and Walters 1997, Malkiel *et al.* 2006, Koski *et al.* 2007, Ohman 2019). Through gut pigment analysis, field studies have confirmed that zooplankton, including many species of copepods, do ingest marine snow (Dagg 1993, Lampitt *et al.* 1993). Experimental studies found that several zooplankton species – including the copepod, *Calanus pacificus* – consumed marine snow aggregates (e.g., Dilling *et al.* 1998), in some cases at rates comparable to that at which they ingested individual phytoplankton cells (Cawley *et al.* 2021). In fact, some species of zooplankton, including the copepod

Oncaea spp., are only able to feed when their food source is in the form of aggregates (Koski *et al.* 2017). Dilling and Brzezinski (2004) found that copepods will ingest marine snow aggregates even in the presence of individual phytoplankton cells, again supporting the idea that marine snow may provide an additional food source in an unpredictable and patchy food environment. Zooplankton feeding on marine snow may impact not only the amount of food consumed but also the composition of their diet, since marine snow aggregates house a rich community of protozoans and microbes (Alldredge and Silver 1988), which are not efficiently captured by mesozooplankton when they are freely suspended in the water column (Alldredge 1972, Lampitt *et al.* 1993). Additionally, zooplankton play a substantial role in regulating and interrupting the downward export of organic carbon (Legendre and Rivkin 2002) through the ingestion and disaggregation of these carbon-rich sinking particles (Karl *et al.* 1988, Dilling and Alldredge 2000, Goldthwait *et al.* 2004, De La Rocha and Passow 2007). In fact, Taucher *et al.* (2018) found that reduced aggregate formation was related to high copepod abundances. While fragmentation of aggregates into smaller particles can result in slower sinking speeds (Alldredge and Gotschalk 1988, Goldthwait *et al.* 2004, Prairie *et al.* 2019, Briggs *et al.* 2020), ingestion and repacking of the carbon into dense fecal pellets can increase the rate of carbon export (Bruland and Silver 1981, Shanks and Edmondson 1989).

Zooplankton grazing is intimately connected to behavior, since patterns of consumption and prey types are strongly influenced by the behaviors exhibited by

a given species, such as cruising, sinking, and jumping (Kiørboe 2011). In addition, planktonic animals will typically use one swimming behavior when searching for food and then switch to another swimming behavior once they have located a food patch (Price 1989, Tiselius 1992, Leising and Franks 2002). In a modeling study, Leising and Franks (2000) concluded that behavioral changes allowing grazers to remain in food patches led to increased foraging efficiency, further suggesting that the existence of highly concentrated food patches may be essential for copepods to meet their daily feeding requirements. Additionally, Tiselius *et al.* (1997) found that the copepod species *Acartia clausi* can significantly reduce the danger of predation by remaining in a food-rich environment, since non-feeding, sinking copepods adjust their vertical position with stronger jumps that are more likely to be detected by predators through hydrodynamic sensing. While patches of highly concentrated food allow for elevated feeding opportunities, copepods, among other zooplankton, have been observed leaving patches with maximum food availability before reaching satiation if, for example, predation risk becomes too high to justify remaining in food patches (Napp *et al.* 1988, Tarling *et al.*, 2002). Copepods may make several short feeding ‘forays’ into patches of high food concentration that increase feeding success while decreasing mortality (Leising *et al.* 2005). While all foraging behavior comes with associated risks (e.g., predation, high energy expenditure, etc.), not all are equally risky or metabolically expensive (Kiørboe 2011), and different feeding behaviors may be observed depending on the type, amount, and distribution of food available.

Based on field observations of zooplankton associated with phytoplankton thin layers (McManus *et al.* 2003), it has been predicted that marine snow thin layers may also be hotspots for zooplankton foraging (Alldredge *et al.* 2002, Prairie *et al.* 2015). Möller *et al.* (2012) observed accumulations of copepods at marine snow thin layers with high resolution imaging, suggesting that these layers may represent a region of enhanced grazing. Other recent studies have also observed an overlap between the vertical distributions of zooplankton and the depth distribution of marine snow aggregates and found that there was stronger overlap of zooplankton with marine snow than with Chl-a (Mooney 2021, Whitmore and Ohman 2021). This idea is reinforced by previous findings that many grazers have the ability to locate and remain in regions of high prey concentration in the form of individual phytoplankton cells (e.g., Tiselius 1992). Menden-Deuer and Grünbaum (2006) found that protistan grazers rapidly responded with prolonged accumulations when exposed to phytoplankton thin layers in lab experiments. Similarly, Tiselius (1992) observed that the copepod species *Acartia tonsa*, when in the presence of phytoplankton thin layers, demonstrated a strong ability to remain inside these patches driven by behavioral changes including decreased swimming velocity and a more horizontal swimming direction. These phytoplankton thin layers not only affect the distribution and behavior of zooplankton grazers but have also been confirmed to be the site of enhanced grazing rates by zooplankton in the field (Menden-Deuer and Fredrickson 2010). Thus, assuming prey availability to be homogenous could result in significant errors in estimating grazing rates and carbon export as a result

of these planktonic trophic interactions (Menden-Deuer and Grünbaum 2006). Despite the evidence that zooplankton are able to locate and forage in phytoplankton thin layers, no experimental study has looked at the behavior or ingestion of copepods or other zooplankton in response to thin layers of marine snow. Determining whether there are differences in foraging responses to different marine snow environments will provide insight on how different distributions may impact grazing and subsequently carbon export in coastal environments.

In this experimental study, we investigated how different distributions of marine snow aggregates affect the foraging behavior of the copepod, *Calanus pacificus*. Stereoscopic imaging was used to compare the vertical distribution of copepods foraging in a thin layer of aggregates and a homogenous distribution of aggregates. By reconstructing the 2D and 3D swimming tracks of the copepods, we quantified different behavioral properties in terms of velocity, path linearity (a measure of how tortuous a copepod's path is), and jump frequency. We also used gut content analysis as a proxy for ingestion to determine the effect of marine snow distribution on copepod ingestion. Based on previous studies showing an association between zooplankton and particle abundance (Möller *et al.* 2012), we predicted that copepods would exhibit behavioral changes when exposed to a marine snow layer, and that residence time and ingestion of marine snow would be enhanced in the layer treatment.

2.2 Methods

During the autumn of 2020, we conducted four experiments in which copepods of the species *Calanus pacificus* were exposed to four different feeding environments (treatments). These four treatments were: a tank with a marine snow layer, a tank with a homogenous distribution of marine snow, and two control treatments without food – one with a density gradient and one with no density gradient (Figure 1). Copepods were recorded with two cameras, allowing us to reconstruct 2D and 3D copepod tracks. From these tracks, we determined vertical distributions of copepods and quantified behavioral properties, including swimming velocity, a measure of path linearity, and jump frequency. We also measured copepod ingestion in the four treatments using gut pigment analysis.

2.2.1 Copepod Collection

Calanus pacificus, a copepod species known to ingest marine snow (Cawley *et al.* 2021), was collected between 11 and 22 days prior to each experiment. The copepods were collected in La Jolla Canyon which is located off the coast of La Jolla, CA (32° 51.720' N, 117° 16.816' W). A 333 µm mesh plankton net was towed at a depth of about 150 m for copepod collection. The contents of each tow were diluted and chilled, and then sorted for the adult female stage of the species *C. pacificus*.

Prior to the experiment, the copepods were kept in complete darkness in an incubator at 18°C. Every other day, the beakers were cleaned by replacing old seawater with fresh filtered seawater, and the copepods were fed *Thalassiosira*

weissflogii. Twenty-four hours prior to each experiment, a total of 120 copepods were starved (30 copepods for each treatment) to ensure equal gut content for all individuals (Tiselius 1992, Dilling and Brzezinski 2004). The copepods for each treatment were placed in separate 100 mL beakers filled with filtered seawater and stored in complete darkness at room temperature (~21 °C) until just prior to the experiment. For each treatment, the copepods were starved in filtered seawater with a density equal to that of the top layer fluid for that treatment, which differed between treatments because of how each feeding environment was created (Table 1). This allowed for the copepods to be acclimated to the density of the top layer fluid in their assigned treatment tank to limit any shock or behavioral reaction at the beginning of the experiment.

2.2.2 Phytoplankton Cultures and Aggregate Formation

Cultures (non-axenic) of the diatom species *T. weissflogii* (CCMP1050, obtained from the National Center for Marine Algae and Microbiota) were grown in f/2 media as a maintenance culture. This species of chain-forming diatom was chosen because it is known to form aggregates in lab settings (Grossart *et al.* 2006, Prairie *et al.* 2019).

Sixteen days prior to each experiment, four identical cultures of *T. weissflogii* were started with a concentration of 10,000 cells/mL in 1.8 L of f/2 media (two each for the layer treatment and the homogenous treatment). These cultures were kept on a 12:12 hour light:dark cycle at room temperature and phytoplankton concentration was measured every other day with a particle

counter (Beckman Coulter). The phytoplankton cultures were grown for a total of thirteen days, corresponding to roughly the middle of their exponential growth phase; previous experiments have shown that aggregates at early exponential and late exponential growth phases are readily ingested by *C. pacificus* (Cawley *et al.* 2021).

After the phytoplankton cultures grew for thirteen days, and three days prior to the experiment, each *T. weissflogii* culture was diluted to 32,500 cells/mL and transferred into a cylindrical acrylic tank with a volume of 2.2 L and circumference of 51 cm. The cylindrical tanks used to form aggregates designated for the layer treatment were filled with seawater with density equal to the top layer fluid for that treatment (Table 1). The tanks used to form aggregates designated for the homogenous treatment were filled with seawater that had a density $\sim 0.0020 \text{ g/cm}^3$ less (i.e., a salinity of ~ 1.3 psu less) than the fluid for that treatment; this was done to allow for slower aggregate settling speeds in that treatment, based on observations in preliminary experiments. The cylindrical tanks were placed on a roller table and were allowed to rotate at a speed of 4.3 RPM for 3 days in the dark to form aggregates, which is a method commonly used to form aggregates in the lab (Shanks and Edmundson 1989).

2.2.3 Foraging Experiments

A single experiment consisted of a set of four different treatments. These treatments were done one after the other, in the following order: control with gradient, layer, homogenous, and control with no gradient. The four treatments

were all created in a rectangular acrylic tank of dimensions 10 cm x 10 cm x 50 cm. In each treatment, 25 copepods were allowed to feed in the tank for between 4-8 minutes (see Table 1).

The control with gradient treatment and the layer treatment required the formation of a density gradient. Because marine snow aggregates decrease their sinking velocity at density discontinuities (Prairie *et al.* 2013), this density gradient allowed for the formation of an ephemeral marine snow layer (lasting about 8 minutes) in the layer treatment. An identical density gradient was created in the control with gradient treatment so we could account for any potential changes in copepod behavior in response to the change in density. Filtered seawater was used for the bottom layer fluid, which had a density between 1.0233-1.0236 g/cm³ (salinity between 33.6-34.0 psu) at room temperature, ~21 °C (see Table 1). Filtered seawater was diluted with DI water to a target density of 0.0040 g/cm³ less (or salinity of 5.3 psu less) than the bottom layer fluid to create the top layer fluid (see Table 1). These densities were chosen based on preliminary experiments by Cayson (2018) that showed that *C. pacificus* did not show adverse reactions (e.g., shock from salinity differences) or substantial behavioral changes when exposed to seawater in this salinity range (28.2-33.6 psu). To form the density gradient, we filled the tank with bottom layer fluid to ~25 cm from the bottom of the tank (approximate halfway mark). In order to get rid of any bubbles adhering to the sides of the tank, which might obstruct a clear camera view, we ran a sponge along the inside tank walls. Then, top layer fluid was carefully poured on top of the bottom layer fluid through a diffuser made

from a sponge that had been soaked in top layer fluid. This diffuser floats at the surface of the water column and prevents mixing at the density interface as the less dense top layer fluid is added to the tank slowly with a pump (Micropump Model GB-P23.JVS.A.B1). This process has been shown to effectively create sharp density transitions in lab settings (Prairie *et al.* 2013). The homogenous treatment and the control with no density gradient treatment did not require a density gradient, and instead were filled entirely with undiluted filtered seawater (i.e., bottom layer fluid from the other treatments).

Just prior to starting each treatment, the starved copepods that were set aside for each treatment were transferred from their 100 mL beakers into 10 mL beakers. This made it possible to pour the entire beaker into a ladle which was used to transfer the copepods into the tank. For treatments that required marine snow aggregates (the layer treatment and the homogenous treatment), the cylindrical tank containing aggregates was carefully taken off the roller table and placed upright so that the aggregates slowly settled to the bottom of the tank. The marine snow aggregates were then carefully transferred into a small glass vial, so as not to break up the fragile aggregates, such that the total volume of aggregates in the vial was equal to roughly 5 mL.

Once the tank was set up, the copepods and aggregates were added accordingly, based on the treatment. For both control treatments, the copepods were placed into the tank by transferring them with a ladle, which was gently placed at the surface of the water and tilted so the copepods were released into the tank. Transferring the copepods with a ladle ensures little to no disturbance to the

copepods which might influence their behavior (Dagg 1993), and also ensures that they do not have a biased downward velocity (due to the jet produced by the pipette). For the layer treatment, the aggregates were pipetted into the tank just below the surface, such that they had a relatively even horizontal distribution. The copepods were ladled into the tank once a distinct marine snow layer had formed. For the homogenous treatment, about half of the aggregates were pipetted just below the surface into the tank, again such that the distribution of aggregates horizontally was fairly homogenous. Then all 25 copepods were ladled into the tank, with the remaining aggregates added afterwards. This method created a roughly homogenous distribution of aggregates sinking around the copepods throughout the time of camera recording.

The experimental tank was set up on a table and was lit from below with a near-infrared light-emitting diode (LED) aimed upwards through a Fresnel lens through a cut-out in the table with an overlying piece of clear plexiglass. We used a near-infrared light so that the copepods' behavior was not affected by the light source (Tiselius 1992), while still allowing for the tank to be illuminated in a way that the cameras could record the copepods and aggregates. Two near-infrared sensitive cameras (Point Grey Grasshopper Camera Model GS3-U3-41C6NIR-C) were set up at 90° angles facing two neighboring sides of the tank. During the experiments, the cameras recorded at 12 frames per second. The recording started immediately prior to adding the marine snow aggregates to the tanks (or the copepods in the case of the control treatments). For the two control treatments, the recording was stopped once the copepods had been in the tank for approximately

8 minutes. For the layer treatment, the recording was stopped once the aggregates started falling out of the layer. For the homogenous treatment, the recording was stopped once the aggregates started sinking out of the bottom of the field of view to limit the amount of time that the animals may be feeding on aggregates sitting on the bottom of the tank (see Table 1 for duration of each treatment in each experiment, defined as the time between when the first copepod entered the field of view of the camera and when the cameras were stopped). The images had a field of view of ~30 cm x 10 cm. This field of view is vertically centered, so that it is ~10 cm from the bottom of the tank and ~10 cm from the top of the tank. The density gradient, which is located ~25 cm from the bottom of the tank, is located at roughly the halfway mark in the field of view (~15 cm from both the bottom and top of field of view).

Once copepod behavior had been recorded, the cameras were turned off and the copepods were carefully siphoned out of the tank onto a 100 μ m mesh sieve. All the water from the tank was saved in a bucket for filtering after the experiments. Copepods were then collected for gut pigment analysis. After the copepods were removed from each treatment tank, using both cameras we recorded images of a ruler aligned vertically in the center of the tank, which were used for image calibration.

2.2.4 Gut Pigment Analysis

After the copepods were siphoned out of the tank and onto the sieve, pairs of copepods were added to amber vials filled with 3mL of 90% acetone (10 per

treatment), recovering 20 of the copepods in each treatment. Copepods in each vial were sonicated (Qsonica Sonicator Model CV334) at 40% amplitude for 5 seconds to break up the organisms and release gut content into the acetone. The amber vials were then placed in a -20°C freezer overnight.

The water of each treatment tank was also filtered and analyzed for chlorophyll *a* concentration to verify that the control treatments contain close to no chlorophyll *a* concentration and to determine any differences in food concentration between the layer treatment and homogenous treatment. Prior to filtering, the water from each tank was well mixed, and then three subsamples of 25 mL each were filtered onto a GF/F filter. These filters were then placed in amber vials filled with 5 mL of 90% acetone to extract the chlorophyll *a* into solution and the vials were placed into a -20°C freezer overnight.

The following day, the copepod gut and tank water samples were analyzed using a fluorometer (Trilogy, Turner Designs) to measure the concentration of total pigment (chlorophyll *a* and phaeophytin combined) in the acetone solution. The copepod sample measurements represent the gut pigment content of the copepods from the experiments. We calculated the concentration of total pigment per copepod (G , in units of $\mu\text{g}/\text{copepod}$) using the equation (Dam and Peterson 1988):

$$G = \frac{F_s \left(\frac{r}{r-1} \right) (R_b - R_a) * (L) * DF}{n} \quad (1)$$

where F_s is the response factor of the fluorometer, r is the before-to-after acidification ratio of a pure chlorophyll *a* solution, R_a and R_b represent the

fluorescence readings before and after acidification, L is the volume of extract (0.003 L of acetone) prepared before dilution, DF is the dilution factor, and n is the number of copepods per vial. Samples with the GF/F filters with the treatment tank water were similarly measured for concentration of total pigment using EPA Method 445.0.

To normalize the copepod gut content measurements in the layer treatment and homogenous treatment, we first subtracted the average gut content across both control treatments, and then divided by the total time (in hours) that the copepods were exposed to food (using the experimental durations in Table 1) to account for differences in duration between the layer treatment and homogenous treatment.

2.2.5 Constructing Copepod Tracks

All images in every treatment were processed in MATLAB. The images were cropped horizontally to remove the tank walls on the left- and right-hand sides of the image which can interfere with tracking copepods. Once the images were cropped, an image from before any copepods or aggregates were added to the tank was subtracted from all other images to remove constant background parts of the images (e.g., tank walls, air bubbles, etc.).

Since the copepods and aggregates are light against a dark background, we applied a threshold to the images with which we could identify copepods and aggregates; a group of connected pixels over a defined threshold were identified as a potential copepod or aggregate. Copepods were manually identified by eye

when they first entered the field of view, and their x- and y-coordinates in the first image they appeared were used to initiate the tracking of each individual.

Copepod tracks were then created for each camera separately by matching pixels identified as copepods in subsequent images (Figure 2). Copepods were matched by minimizing the change in distance between individual copepods in neighboring frames, and accounting for a maximum distance that can be traveled between frames. In some cases, copepod tracks were not able to be constructed automatically through this process because of the close proximity to other copepods or aggregates. In these cases, copepods were either manually tracked for a period of time until they could be automatically tracked again, or that track was terminated and a new track was created if and when the copepod reappeared. Copepods that exited through the bottom or top of the field of view or that would swim too near to the wall would also result in those tracks being terminated.

The copepod track coordinates were linearly converted from pixels to centimeters using the image of a ruler taken immediately following each treatment, defining copepod vertical location as distance (in cm) from the bottom of the tank. The 2D constructed tracks from only the first camera were used to illustrate the vertical distribution and movement of the copepods over time for each treatment. Corresponding tracks from both cameras were then aligned to construct 3D copepod tracks. The tracks were matched by visually pairing a track from each camera based on similar locations over time along the z-axis (vertical dimension), which is shared between the two cameras (Figure 3). For the 3D copepod tracks, the x and y positions were obtained from the horizontal positions

in each camera, respectively, while the z positions were obtained from the vertical positions in the first camera.

2.2.6 Determining Vertical Distributions of Copepods

Vertical distributions of copepods in each treatment were assembled every 5 seconds in discrete depth bins (in 3 cm intervals). This was done by using all 2D tracks from each treatment (across all 4 experiments) and determining the number of copepods in each given depth bin at each time. Although 2D tracks were constructed for both Camera 1 and Camera 2, only the tracks created with images from Camera 1 were used in any analysis requiring the 2D tracks. In all treatments, copepods that were not visible because they were above or below the field of view or too close to the wall were excluded from these vertical distributions. However, in the layer treatment, copepods were also not visible when they were within the layer of aggregates, which obscured the view of any copepods (Figure 2A). Given this, each copepod was tracked until it entered the layer and then a new track was started for any copepod leaving the layer. Thus, the vertical distributions for the layer treatment determined solely from the 2D tracks did not accurately represent the number of copepods in the middle depth bin (corresponding to the location of the layer). To account for this, we recorded every time point that a copepod entered and disappeared into the marine snow layer (from both above and below the layer) and every time point that a copepod emerged from the marine snow layer (from both above and below the layer). We then added a copepod to the middle depth bin (corresponding to the location of

the layer) for every time point that a copepod entered the layer and subtracted a copepod from that depth bin for every time point that a copepod emerged from the layer. Another issue was that more copepods were observed entering the layer than emerging from the layer in all experiments. This may have occurred if a copepod swam to the wall while inside of the layer (something that we visually observed while running the 2D track construction program), and so we could not observe that individual when it emerged from the layer. To address this issue, we took the average of the time points when copepods emerged from the layer and added that value as additional emerging times such that the number of time points for copepods entering the layer and emerging from the layer were equal for each experiment. The vertical distributions created from this method were both displayed over time, and through discrete histograms at specific time points (30, 70, 110, and 150 seconds) between treatments.

The 2D tracks were also used to calculate residence times within a vertical section of the tank surrounding the layer, defined as the ‘layer region’. This layer region was defined as the vertical section of the tank spanning 4 cm above the layer to 4 cm below the layer. Layer depth, measured in terms of distance from the bottom of the tank, varied slightly across the experiments (Experiment 1: 24.4 cm, Experiment 2: 24.1 cm, Experiment 3: 25.0 cm, Experiment 4: 24.6 cm). For each experiment, the layer depth in the layer treatment was also used to define the same layer region in the other treatments. Since continuous copepod tracks could not be constructed through the marine snow layer in the layer treatment, it was not possible to calculate the residence time for an individual copepod. Instead, we

recorded all times that the copepods entered the layer region and all times that copepods left the layer region, omitting all times in which copepods entered the layer for less than 5 seconds before leaving. Average residence time for that treatment and experiment was then calculated as the average entering time subtracted from the average leaving time. To validate this process, we also measured residence time for each individual for all treatments that the copepod tracks were not interrupted by the presence of an aggregate layer (homogenous, control with gradient, control with no gradient). When comparing the average residence time of the individuals with the average residence times calculated from the entering and leaving times, the values were close (with average residence times between the two methods differing by 11%, on average), supporting the use of this method.

2.2.7 Calculating Copepod Behavioral Properties

We used the 2D and 3D tracks to quantify five metrics of copepod behavior: vertical velocity, jump frequency, velocity, and a measure of path linearity both vertically and overall. We calculated vertical velocity, jump frequency, and vertical path linearity from the 2D tracks, (since these metrics only depend on changes in the vertical dimension), while velocity and overall path linearity were calculated from the 3D tracks. The velocity of a copepod over time was calculated by dividing the total distance a copepod moved between subsequent images and dividing by the time between images. Similarly, vertical velocity was calculated by dividing the change in vertical location (positive

defined as downward) between subsequent images by the time between images. Jump frequency was calculated by dividing the number of copepod jumps by the total time of the copepod track. Copepod jumps were defined as distinct times when a copepod moved upwards at a rate of 0.2 cm/second (a little less than one female copepod body length per second), which was a value found to detect most jumps. Given our frame rate of 12 frames per second, if a jump lasted for less than 1/12 of a second, it might not be detected; however, we found that the majority of jumps occurred over the span of more than one image (17-43% of jumps had a duration of just one image on average, varying by treatment). The path linearity of a copepod was calculated as the net gross displacement ratio (NGDR), which is found by dividing the net distance travelled by the gross distance travelled (Buskey 1984). NGDR was calculated as a running average over 2 seconds. The values of NGDR lie between 0 and 1, where values closer to 0 signify greater turn frequencies, while values closer to 1 signify a more linear path (Dur *et al.* 2011). Vertical path linearity (hereafter referred to as vertical NGDR) was calculated in the same way (also as a running average over 2 seconds) but using the net distance travelled and gross distance travelled only in the vertical direction; this metric can be used to identify the extent to which a copepod changed its vertical swimming direction as it moved through the tank (with a value 1 indicating that a copepod never changed directions vertically). For all five behavioral properties, we took the average value for each track, but excluded values from any tracks that were less than 10 seconds long. In order to determine differences in copepod behavior near the layer, we also examined behavioral properties only within the

layer region (defined the same way as for the calculation of residence time); in this case, tracks that occurred in the layer region for less than 5 seconds were excluded.

2.2.8 Quantifying Aggregate Size and Settling Velocity

To quantify the variability of aggregates in our experiments, we calculated aggregate size (in terms of area) and settling velocity across treatments (layer and homogenous) and across experiments. In the layer treatment, this was calculated from the time between when the aggregates first entered the field of view until just before the first aggregates reached the density gradient. In the homogenous treatment, these aggregate properties were measured from the time between when the aggregates first entered the field of view until just before the copepods entered the field of view. Aggregates were identified in each image as a group of connected pixels over a defined threshold that was above a specified area (0.01 cm²). Aggregates were automatically tracked between images in the same way as copepods, minimizing the change in distance in neighboring frames. If there was no aggregate in the subsequent image within a specified maximum distance, the aggregate track was terminated. Aggregate area was calculated per aggregate track by averaging the number of connected pixels per image and converting to centimeters. Aggregate settling velocity was calculated per aggregate track by averaging the aggregate distance travelled between subsequent images and dividing by the time between images. Aggregate tracks less than 10 seconds in duration were excluded from analysis. The aggregates in the homogenous

treatment for experiments 3 and 4 were very faint in the recorded images and thus difficult to track. As such, aggregate settling velocity and area data were obtained from fewer total tracks in these cases.

2.2.9 Statistical Analyses

A two sample t-test was run to determine differences in the mean normalized copepod gut content between the layer and the homogenous treatment for each experiment. A one-way ANOVA, followed by a Tukey-Kramer *post hoc* test, was run to determine differences in non-normalized copepod gut content across treatments for each experiment.

A Kolmogorov-Smirnov test was run to determine differences between the vertical distributions at discrete time points (30, 70, 110, and 150 seconds) pairwise between treatments. Since this resulted in a large number of tests (24 in total), a lower significance level of 0.005 was used.

A one-way ANOVA, followed by a Tukey-Kramer *post hoc* test, was run to determine differences between the residence time between treatments (with $n=4$ per treatment, using each average value per experiment).

A one-way ANOVA, followed by a Tukey-Kramer *post hoc* test, was run to determine differences in the mean values for each behavioral property (velocity, vertical velocity, jump NGDR, and vertical NGDR) between treatments (pooled for all experiments). Note that, due to the fact that we were unable to track the copepods behind the aggregate layer in the layer treatment, this treatment had approximately twice the amount of tracks as compared to all other

treatments (since tracks were essentially cut in half at the layer) (Table 2).

Because of this, our sample size of copepod tracks for the layer treatment was artificially inflated, by approximately a factor of 2. In order to account for this, we adjusted the degrees of freedom for error downward by half the number of tracks in the layer treatment. Similarly, for Tukey–Kramer *post hoc* comparisons that included the layer treatment, the test statistic was calculated using the adjusted sample size for the layer treatment (reduced by a factor of 2).

2.3 Results

Average ingestion as measured by normalized copepod gut pigment content was higher in the layer treatment than in the homogenous treatment in 3 out of the 4 experiments; however, this difference was only significant in Experiment 2 (Figure 4). When considering raw gut pigment content (not accounting for differences in times between treatments), both Experiment 2 and 4 showed significantly higher average gut content for copepods exposed to the layer treatment (Supplementary Figure 1). Food concentration in the layer treatment and homogenous treatment ranged from 4.625–30.570 $\mu\text{g pigment/L}$, with food concentration sometimes higher in the layer treatment and sometimes higher in the homogenous treatment (Table 2). There was a general trend that copepods had a higher gut content in treatments that contained higher concentrations of food (Table 2, Figure 4)

In the homogenous treatment and control with no gradient treatment, most individuals sank in a general downward direction in the tanks, while some

individuals exhibited different behavior, swimming up in the tank and performing small jumps (Figure 5). In the control with gradient treatment, the copepods exhibited a series of jumps at the halfway point in the tank where the density gradient was located, remaining in the general region of the density gradient (for up to 200 seconds, but varying widely among individuals) rather than sinking directly through it (Figure 5). In the layer treatment, many individuals passed through the layer and then changed their vertical direction to swim back into the layer of aggregates (Figure 5). The swimming patterns observed in the layer treatment and control with gradient treatment resulted in accumulations of copepods in the middle of the tank as demonstrated by the vertical distributions pooled for all four experiments (Figure 6 A, C). Accumulations were not observed in the homogenous treatment and control with no gradient treatment (Figure 6 B, D). At the 30-second time point, there were no substantial qualitative differences in vertical distributions, with the copepods in all treatments spread out relatively evenly across the top half of the tank (Figure 7 A, E, I, M); however, the vertical distributions between all pairs of treatments were found to be significantly different with the exception of the control with gradient treatment and control with no gradient treatment (Table 3). At the 70-second time point, copepods appear at a peak concentration at the halfway point in the tanks in both the layer treatment and control with gradient treatment (Figure 7 B, J), with no such accumulation in copepod distribution observed in the homogenous treatment and control with gradient treatment (Figure 7 F, N). Significant differences in distributions were found between the layer treatment and all other treatments in

addition to between the control with gradient treatment and the homogenous treatment (Table 3). The peak in copepod concentration at the layer depth remained in the layer treatment at the 110-second time point (Figure 7C), with a distribution pattern that was significantly different from all other treatments (Table 3). All other treatments had a somewhat even distribution across the middle to lower half of the tank, with a small peak at ~25 cm depth in the control with gradient treatment (Figure 7 G, K, O). Finally, at the 150-second time point, copepods were no longer distributed with a peak concentration in the middle of the tank in the layer treatment, but rather distributed relatively evenly across the bottom half of the tank (Figure 7D). No significant differences in copepod distribution were observed at 150 seconds between any treatments (Table 3), although a smaller peak in concentration at the ~25 cm depth mark persists in the control with gradient treatment (Figure 7L).

Average residence time within the defined layer region (layer depth \pm 4cm) was found to be significantly higher in the two treatments with a density gradient (layer and control with gradient) compared to the two treatments with no density gradient (homogenous and control with no gradient). No significant difference was found between the layer and the control with gradient treatment or the homogenous and control with no gradient treatment (Figure 8).

The only significant difference between overall velocity in the whole tank across treatments, was that velocity in the homogenous treatment was significantly higher than velocity in the control with gradient treatment in the whole tank (Figure 9A); however, for velocity only within the layer region, more

differences were observed, with velocity in the control with gradient treatment significantly lower than that in the homogenous treatment and control with no gradient treatment (Figure 9B). While there were some differences in vertical velocity in the whole tank across treatments, the vertical velocity only within the layer region varied more substantially between treatments, where vertical velocity in the homogenous treatment was significantly higher than the vertical velocity in all other treatments and vertical velocity significantly higher in the control with no gradient treatment than the control with gradient treatment. Within the layer region average vertical velocity was substantially lower in both the layer treatment and control with gradient treatment between 0.1-0.2 cm/sec lower than in homogenous and control with no gradient (Figure 9 C, D). Similarly, while there were some modest differences in jump frequency between treatments in the whole tank, jump frequency calculated within the layer region was significantly (and substantially) higher in the layer treatment and control with gradient treatment compared to the homogenous treatment and control with no gradient treatment (Figure 9 E, F). There was no significant difference in NGDR between any of the treatments in the whole tank; however, when calculated just within the layer region we found that NGDR in the layer treatment was significantly lower (indicating a more tortuous path) than all treatments except for the control with gradient treatment (Figure 9 G, H). The vertical path linearity across the whole tank for the control with gradient treatment was significantly lower (indicating more reversals of direction) than all other treatments (Figure 9I). Within the layer region, vertical NGDR for the control with gradient treatment was only

significantly lower for the homogenous and control with no gradient treatments and not significantly lower than in the layer treatment; however, vertical NGDR for the layer treatment was significantly lower than the homogenous and control with no gradient treatments (Figure 9J).

Aggregate settling velocity was higher in the layer treatment compared to the homogenous treatment for every experiment, where settling velocity in the homogenous treatment was at most half that of the layer treatment (Figure 10A). Additionally, settling velocity in the homogenous treatment for Experiments 3 and 4 was substantially lower than all other settling velocities (Figure 10A). Aggregate area was also greater across all layer treatments for every experiment (Figure 10B). There was no substantial difference in aggregate size between the layer treatment and homogenous treatment for Experiments 1 and 2, but aggregate size was substantially lower in the homogenous treatment than the layer treatment for Experiments 3 and 4 (Figure 11).

2.4 Discussion

In all experiments, *Calanus pacificus* ingested marine snow aggregates when exposed to both the layer and homogenous distributions of aggregates, confirming active consumption of phytoplankton aggregates by *C. pacificus* that has been found in previous studies (Dilling *et al.* 1998, Cawley *et al.* 2021). Additionally, feeding on aggregates was potentially higher in the layer distribution than the homogenous distribution, although this difference was only significant in one of the four experiments (Figure 4). Our results also showed

accumulations of copepods in the center of the tank occurring in both treatments with a density gradient (layer and control with density gradient) but not in the two treatments with no density gradient (homogenous and control with no density gradient) (Figures 6, 7). This is further supported by the finding that copepods spent significantly more time in the layer region of our tanks in the layer treatment and control with gradient treatment (Figure 8). Finally, differences in swimming properties were observed across treatments, especially when analyzing copepod behavior within the layer region, with jump frequency being higher and vertical velocity being lower in the layer treatment and control with gradient treatment compared to the two treatments with no density gradient (Figure 9 D, F).

2.4.1 Changes in Copepod Behavior and Vertical Distributions in Response to Aggregate Layers

The finding that residence time of copepods in the layer region was higher in both the layer treatment and control with gradient treatment is consistent with the observed behavioral changes exhibited by copepods in this region. Increased jump frequency, decreased vertical velocity, and decreased vertical path linearity (indicating more changes in the vertical swimming direction) allowed the copepods to remain in the layer region for longer periods of time rather than just sink or swim straight through it, as was seen in the homogenous treatment and the control with no gradient treatment. While we did see these differences in behavior between the two treatments with a density gradient and the two treatments without

a density gradient, we did not see substantial differences in behavior or residence time between copepods in the layer treatment and the control with a gradient treatment. This makes it difficult to conclude whether these changes were in response solely to the presence of a sharp change in salinity or additionally to the presence of food.

Copepods can exhibit different types of behavior in response to different physical cues. Woodson *et al.* (2005) looked at responses of two species of copepod, *Acartia tonsa* and *Temora longicornis*, to a salinity-driven density gradient. Copepods of both species either swam along the gradient layer or turned and swam away after initial contact with the gradient, indicating that sharp density gradients may act as barriers to vertical copepod movement, resulting in aggregations at these discontinuities (Woodson *et al.* 2005). A later study with the same copepod species supported this finding, but rarely observed any individuals crossing the density gradient (Woodson *et al.* 2007); by contrast, we observed most, if not all, of the copepods in our experiments crossing the density gradient after performing a series of jumps and/or decreasing their vertical velocity as they swam through the discontinuity. This aligns with a study by Cayson (2018) that shows similarly subtle behavioral responses to density gradients, suggesting that copepods alter their behavior in response to density gradients may depend on the strength of the density gradient and the species of copepod.

Copepods can elicit strong behavioral responses due to chemical cues as well. Studies on chemodetection have shown that copepods exhibit a very distinct swimming behavior when following a chemical trail, such as one left behind by a

mate (e.g., Yen *et al.* 1998); this swimming pattern has been termed casting behavior, in which the copepod casts with equal frequency in all directions to maximize the probability of encountering the trail, resulting in a spiral pattern (Weissburg *et al.* 1998). Lombard *et al.* (2013) observed live copepods exhibiting casting behavior upon encountering an artificial chemical plume, with similar properties to the plume of a sinking marine snow aggregate, supporting the role of chemodetection in the ingestion of marine snow by copepods as had been described in modeling studies (e.g., Kiørboe and Jackson 2001). In our study, there was somewhat decreased average NGDR in the layer treatment, but we did not observe any 3D tracks with dramatically reduced NGDR that would indicate the presence of casting behavior. We did, however, see numerous examples of casting behavior in previous preliminary experiments (Elena Beckhaus, unpubl. data), which may have been in response to tracking a chemical plume from an aggregate. Many factors may determine whether copepods perform casting behavior in the presence of sinking aggregates, including the presence of males (since male copepods were also included in the preliminary experiments), species of copepod, and properties of aggregates. The lack of substantial behavioral differences between treatments and their paired control (i.e., layer vs. control with a gradient and homogenous vs. control with no gradients) does not support behavioral changes primary driven by chemical cues in our experiments.

Woodson *et al.* (2007) proposed a cue hierarchy where a physical change (a velocity gradient, in the case of this study) presented an initial cue for limiting search regions and then chemical cues and responses to contact with food elicit

further responses and behavioral changes to remain within the food patch. While we did not find significantly higher average residence time in the layer treatment than in the control with a gradient treatment, the vertical distributions in the two treatments indicate subtle but noteworthy differences (Figure 7). We observed a stronger initial accumulation of copepods at the layer depth in the layer treatment (as indicated by the number of copepods in the middle depth bin), and there were significant differences in the vertical distributions between the layer treatment and the density with a gradient treatment at both the 70- and 110-second time point. However, most individuals in the layer treatment left the layer region by the 150-second time point, whereas several copepods in the control with a gradient treatment still remained within the layer region at 150 seconds. This suggests that the accumulation of copepods in the presence of density gradient may be weaker but last longer when no food is present. Although the overall path linearity is slightly lower in the layer region of the layer treatment than the control with gradient treatment, the vertical path linearity is lower in the control with gradient treatment, suggesting that the copepods changed their vertical direction more when there was no food present. This might suggest that, without the food signal in the control with gradient treatment, the copepods continued to search in a way that the copepods in the layer treatment did not (Tiselius 1992, Woodson *et al.* 2007). Additionally, one reason that the accumulation in the layer treatment did not seem to last as long, might be because the copepods filled their guts and drifted out of the food patch. Although our experiment is short (lasting approximately 8 minutes), studies have shown that, while there is a lot of

variability in gut passage time between individuals, that gut passage time for a copepod could be as short as 5 minutes (Karaköylü and Franks 2012).

Furthermore, average gut content for the copepods in our experiment is comparable to the gut content of copepods that fed for an hour (Cawley *et al.* 2021).

We must again note that, in our experimental design, the copepods could not be tracked through the layer of aggregates in the layer treatment, and this may affect the vertical distribution data. As such, there is the potential that the differences in copepod distribution at ~25 cm depth between the layer and control with a gradient treatment may not have been less significant had we been able to track individuals through the layer. In addition, behavioral properties within the layer of aggregates could not be measured, and instead were only measured in the region just above and below the aggregate layer. It is possible that, had we been able to incorporate the behavioral data within the aggregate layer, that there may have been greater behavioral differences between the layer treatment and control with gradient treatment. While we could not avoid losing the copepods behind the layer in our experiments, in future studies it could be possible to track copepods within a layer of marine snow by angling the cameras from a top and bottom view.

2.4.2 Understanding our Results in the Context of Natural Coastal Environments

Marine snow, like many other food sources in the water column, can be patchy over a wide range of spatial and temporal scales (Krembs *et al.* 1998,

Franks and Jaffe 2001). A frequently observed example of this spatio-temporal heterogeneity is the formation of marine snow thin layers at density discontinuities in the water column (McIntyre *et al.* 1995, Alldredge *et al.* 2002, McManus *et al.* 2003), which have been suggested as potential hot spots for zooplankton foraging (Möller *et al.* 2012). Our experimental results have shown that copepods of the species *C. pacificus* do alter their behavior at marine snow layers that occur at sharp density gradients, such that their residence time in these food patches is increased. Given the common occurrence of marine snow layers associated with sharp density gradients in coastal waters, these findings have implications for our understanding of how copepod foraging on marine snow aggregates may vary in different regions of the ocean.

Because this was an experimental study, there were important differences between the marine snow layers in our tank and the ones in natural environments. For one, we formed our density gradients by creating a sharp change in salinity, which has been shown to successfully result in the formation of a temporary marine snow layer in an experimental setting (Prairie *et al.* 2013). Density gradients in coastal waters are, however, more commonly driven by sharp changes in temperature. Thus, the thin layer that we created in our experiments would more likely share the characteristics of one occurring in a shallow Pacific fjord (salinity-driven), as observed by Alldredge *et al.* (2002), than one occurring in non-estuarine coastal regions (temperature-driven). Furthermore, the thin layer formed in our experiments was about 1 cm thick, extended horizontally over 10 cm, and persisted for ~ 8 minutes, while thin layers observed in the field have

been observed to be up to 3.5 m thick, horizontally extend over kilometers, and persist for days (McIntyre *et al.* 1995). Additionally, while marine snow is ubiquitous throughout the water column, its abundances can range between <1 and 100 aggregates in a liter of seawater (Simon *et al.* 2002), and zooplankton foraging rates are likely to vary with aggregate abundance in turn. Although we had aimed to keep food concentration consistent across all experiments for the layer treatment and the homogenous treatment, the differences we found between treatments from post-experiment measurements (Table 2) may partially explain the patterns in copepod ingestion we observed (Figure 4).

There are many other factors that may affect copepod behavior that are not represented in our experimental set up. For one, the copepods were not under the threat of predation in our tanks. Kiørboe and Thygesen (2001) propose that a copepod's residence time attached to an aggregate is shortened by a heightened predation risk of staying in place. Thus, copepod foraging behavior might be altered in our experiments by the absence of optimizing trade-offs between feeding and risking predation. McManus *et al.* (2003) also suggests that in situ vertical distributions of zooplankton around a persistent thin layer may be impacted by vertical migration patterns of zooplankton. While we kept the copepods in complete darkness for 24 hours prior to the experiment and throughout the entirety of the experiment, bringing the animals into a dark room may have triggered an increase in feeding activity resulting from changes in their endogenous rhythms (Tiselius *et al.* 1995). There could also be effects from starving the copepods for 24 hours prior to experiments to clear their gut, since

Tiselius (1992) found that severely starving (24 hours) copepods had a significant effect on their motility. Starved copepods may be particularly responsive to food signals and increase their foraging rates (Lombard *et al.* 2013). Finally, copepods are not exposed to walls or bottoms in their natural environment, and thus their behavior is likely altered when encountering the walls and bottom of our experimental tank; however, we attempted to minimize the effect of this in our data analysis by not tracking copepods near walls of the tanks.

Lastly, future studies could consider further underlying factors driving copepod mechanosensory and chemosensory responses to marine snow. As mentioned previously, aggregate properties are likely to affect the ability of copepods to hydromechanically or chemically detect these particles. The aggregates that we formed for our experiments are much denser than those naturally formed in the ocean (Shanks 2002, Prairie *et al.* 2013). Denser aggregates sink faster and thus leave behind a more concentrated, but very long and thin plume, which decreases the chance that copepods may encounter the plume and also makes it more difficult to track (Kjørboe and Thygesen 2001). Aggregates in our experiments had high settling velocities, especially in the layer treatment (since aggregates in the homogenous treatment were formed in lower density seawater in order to intentionally slow their sinking rate) (Figure 11). These high settling velocities may have made the aggregates more difficult to chemically track and explain why we did not observe copepods exhibiting casting behavior; however, copepod swimming velocity in our experiments were observed to be as high as or higher than the settling velocities of aggregates,

suggesting that a lack of casting behavior was not due to a copepod's inability to catch up the sinking aggregates. While not the goal of our experiments, future studies should be conducted with sinking aggregates of different densities to examine how copepod behavior, in particular tracking the plume via casting behavior, may be affected by aggregate properties. Furthermore, the water in our experimental tank was not impacted by any disturbances, such as wind- and current-driven turbulence, that are typical of field conditions. Strong turbulence in the water column would dissipate a plume and thus prevent or limit remote chemical detection by zooplankton (Kiørboe and Thygesen 2001). As such, we might expect the copepods in our experimental tank to have a higher success rate at detecting chemical plumes and tracking sinking aggregates than in natural, turbulent conditions. However, turbulence varies spatially and temporally in the ocean and many parts of the ocean can be more quiescent (Franks *et al.* 2022), so our experimental study could be an accurate representation of copepod foraging in weak turbulence leading to low plume dissipation rates. In particular, since turbulence decreases with depth, zooplankton may have more success in chemical detection of aggregates in deeper waters than in the surface layers of the ocean (Jackson and Kiørboe 2004). Examining the effects of turbulence and other environmental parameters could further lend to our understanding of copepod behavior surrounding marine snow in field conditions.

2.4.3 Implications for Plankton Ecology

While other experimental studies have shown that copepods increase their residence time and alter their swimming properties in phytoplankton layers (Tiselius 1992, Menden-Deuer and Grünbaum 2006), this is the first study to compare individual behavior of copepods within a marine snow layer and within a homogenous distribution of marine snow. Understanding how copepods are able to utilize these layers of marine snow as a concentrated food source allows us to make more accurate estimates of copepod distributions and grazing rates in the coastal environments in which marine snow thin layers are common.

Differences in foraging rates between homogenous and layered distributions of marine snow could impact the efficiency of the biological carbon pump and the pelagic ecosystem as a whole. Small-scale interactions, such as the consumption, repackaging, and fragmenting of marine snow aggregates by zooplankton, could impact the rate of carbon export to the deep ocean (Turner 2015). Furthermore, as zooplankton track their food source, their distribution will subsequently be affected, and thus they could exhibit the same patchy distributions as marine snow aggregates. Such spatial heterogeneity in zooplankton populations has important implications for the structure and function of planktonic ecosystems, in terms of productivity, nutrient cycling, and trophic interactions (Pinel-Alloul 1995). Importantly, the distribution of zooplankton will alter the distribution of other pelagic organisms, like their mobile prey, competitors, and predators (Mackas *et al.* 1985), including important commercial fishery species. Thus, the foraging ability of zooplankton in different marine snow

environments affects both carbon cycling in the ocean and the pelagic food web, and it provides another example of how fine-scale interactions can have an effect on global processes.

Table 1. Experimental details for each experiment number and treatment, including duration of experiment, number of 2D tracks (from Camera 1) and 3D tracks >10 s in duration, and densities of seawater (and corresponding salinities at 21°C) used in tanks (TLF=Top Layer Fluid, BLF=Bottom Layer Fluid, ARF=Aggregate Rolling Fluid, ETF=Entire Tank Fluid).

Exp. #	Treatment	Duration of Experiment (sec)	# of 2D tracks	# of 3D tracks	Tank Water Density (g/cm ³); Salinity (psu)
1	Layer	502	47	27	TLF = 1.0193; 28.2
					BLF = 1.0233; 33.6
					ARF = 1.0193; 28.2
	Homogenous	169	24	20	ETF = 1.0233; 33.6
					ARF = 1.0223; 32.2
	Control with Gradient	435	32	14	TLF = 1.0193; 28.2
					BLF = 1.0233; 33.6
	Control with no Gradient	490	18	19	ETF = 1.0233; 33.6
2	Layer	496	52	34	TLF = 1.0193; 28.2
					BLF = 1.0233; 33.6
					ARF = 1.0193; 28.2
	Homogenous	209	32	13	ETF = 1.0233; 33.6
					ARF = 1.0223; 32.2
	Control with Gradient	481	36	21	TLF = 1.0193; 28.2
					BLF = 1.0233; 33.6
	Control with no Gradient	481	22	19	ETF = 1.0233; 33.6
3	Layer	470	48	20	TLF = 1.0193; 28.2
					BLF = 1.0233; 33.6
					ARF = 1.0193; 28.2
	Homogenous	313	23	10	ETF = 1.0233; 33.6
					ARF = 1.0223; 32.2
	Control with Gradient	451	25	12	TLF = 1.0193; 28.2
					BLF = 1.0233; 33.6

	Control with no Gradient	439	21	15	ETF = 1.0233; 33.6
4	Layer	463	43	30	TLF = 1.0196; 28.6
					BLF = 1.0236; 34.0
					ARF = 1.0196; 28.6
	Homogenous	209	17	9	ETF = 1.0236; 34.0
					ARF = 1.0226; 32.6
	Control with Gradient	466	21	13	TLF = 1.0196; 28.6
					BLF = 1.0236; 34.0
	Control with no Gradient	413	25	14	ETF = 1.0236; 34.0

Table 2. Post-experiment mean concentration of food (as measured by fluorometer in total pigment) \pm standard error for each experiment and treatment.

Experiment Number	Treatment	Food Concentration in Tank ($\mu\text{g pigment/L}$)
1	Layer	21.72 ± 0.20
	Homogenous	26.50 ± 0.42
	Control with Gradient	0.20 ± 0.04
	Control with no Gradient	0.14 ± 0.02
2	Layer	30.57 ± 0.25
	Homogenous	19.92 ± 0.07
	Control with Gradient	0.11 ± 0.01
	Control with no Gradient	0.13 ± 0.01
3	Layer	12.20 ± 0.32
	Homogenous	23.87 ± 0.31
	Control with Gradient	-0.01 ± 0.00
	Control with no Gradient	0.02 ± 0.01
4	Layer	17.66 ± 0.36
	Homogenous	4.63 ± 0.26
	Control with Gradient	0.14 ± 0.01
	Control with no Gradient	0.13 ± 0.00

Table 3. *P*-values for Kolmogorov-Smirnov (K-S) tests comparing copepod vertical distribution at different set time points (30, 70, 110, and 150 seconds) between each pair of the four treatments (L=Layer, H=Homogenous, CG=Control with a Gradient, CNG=Control with no Gradient). Asterisks represent significant differences (based on a 0.005 significance level).

Time Point (sec)	P-Values from K-S Tests
30	L-H: $P < 0.001^*$ L-CG: $P < 0.001^*$ L-CNG: $P < 0.001^*$ H-CG: $P < 0.001^*$ H-CNG: $P < 0.001^*$ CG-CNG: $P = 0.137$
70	L-H: $P < 0.001^*$ L-CG: $P < 0.001^*$ L-CNG: $P < 0.001^*$ H-CG: $P < 0.001^*$ H-CNG: $P = 0.023$ CG-CNG: $P = 0.015$
110	L-H: $P < 0.001^*$ L-CG: $P < 0.001^*$ L-CNG: $P = 0.003^*$ H-CG: $P < 0.001^*$ H-CNG: $P = 0.116$ CG-CNG: $P = 0.012$
150	L-H: $P = 0.212$ L-CG: $P = 0.018$ L-CNG: $P = 0.069$ H-CG: $P = 0.189$ H-CNG: $P = 0.944$ CG-CNG: $P = 0.225$

Figure 1. Experimental design showing the four treatment tanks: Layer, Homogenous, Control with Gradient (CG), and Control with No Gradient (CNG).

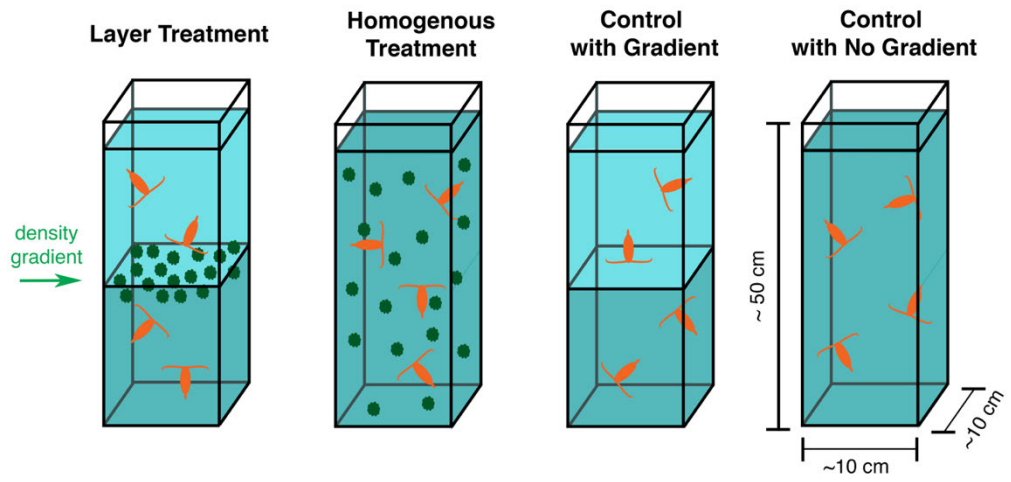


Figure 2. Series of example images in the layer treatment (Exp. 1) demonstrating how copepods were tracked between subsequent images, where (A) shows the whole tank and (B-F) show an inset of the tank (shown in yellow in A) of every third image (separated in time by 0.25 seconds). An example copepod being tracked as it is moving through the tank is circled in green. Axes represent dimensions of tank in the vertical and horizontal direction (in cm).

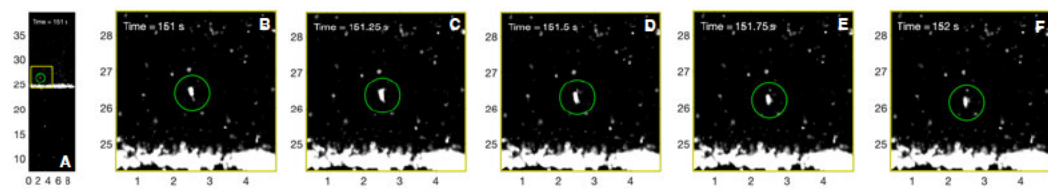


Figure 3. Example of 3D track construction from two 2D tracks in the control with no gradient treatment of Experiment 2. (A) and (B) show the horizontal and vertical trajectory of the 2D track in camera 1 and camera 2, respectively, (C) shows the trajectory in the vertical direction (z-axis) of both cameras vs. time, and (D) shows the resulting trajectory of the 3D track after it is assembled from both cameras.

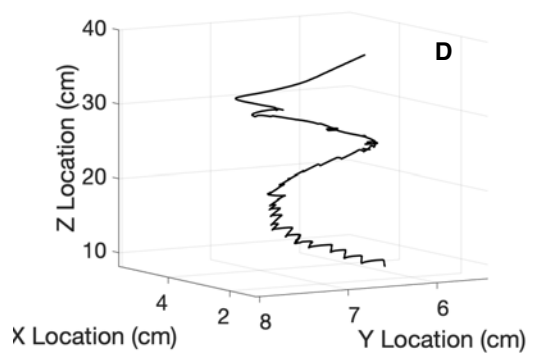
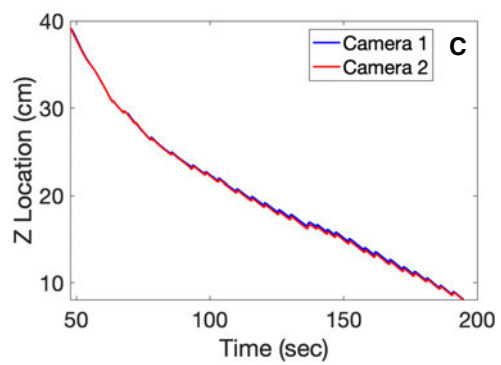
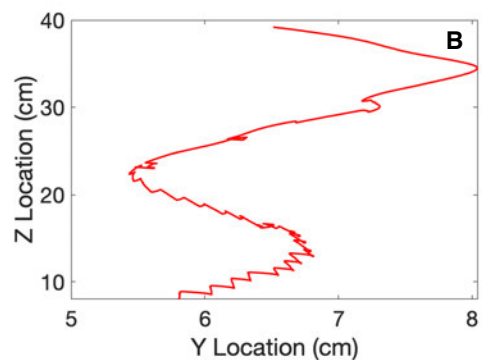
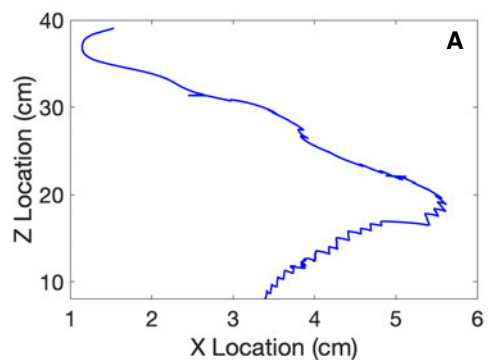


Figure 4. Bar graphs showing average normalized gut pigment content between the layer treatment and homogenous treatment for (A) Experiment 1, (B) Experiment 2, (C) Experiment 3, and (D) Experiment 4. Error bars represent standard error. Two sample t-tests for show no significant difference between treatments for Experiments 1, 3, and 4 (A: $P=0.272$, C: $P=0.390$, D: $P=0.123$), but do show a significant difference between treatments for Experiment 2 (B: $P=0.025$).

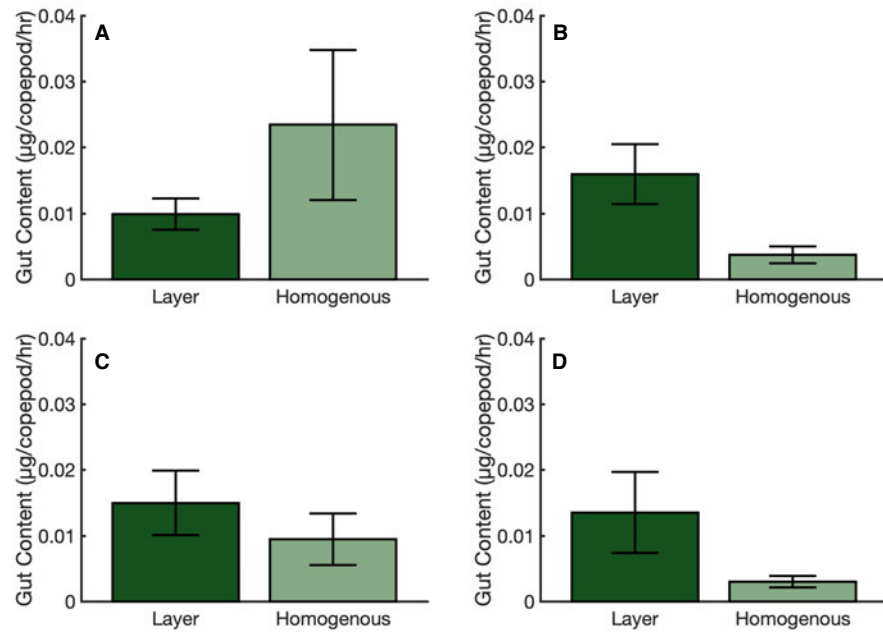
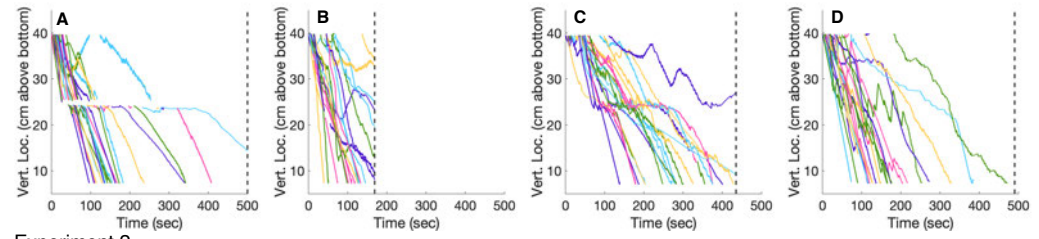
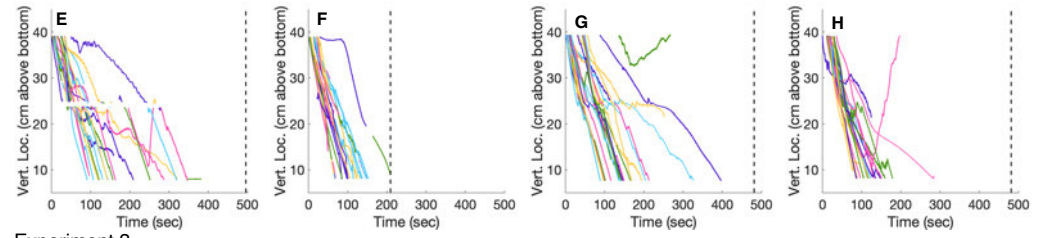


Figure 5. 2D tracks of copepod vertical location over time across all four treatments in each experiment (Experiment 1: A, B, C, D; Experiment 2: E, F, G, H; Experiment 3: I, J, K, L; Experiment 4: M, N, O, P) with the treatments from left to right: layer (A, E, I, M), homogenous (B, F, J, N), control with gradient (C, G, K, O) and control with no gradient (D, H, L, P). Dashed black line represents time at which the cameras were stopped for each treatment. Colors are arbitrary, representing different copepod tracks. Note that the empty spaces between tracks at ~25 cm in the layer treatment are a result of not being able to track copepods through the marine snow layer.

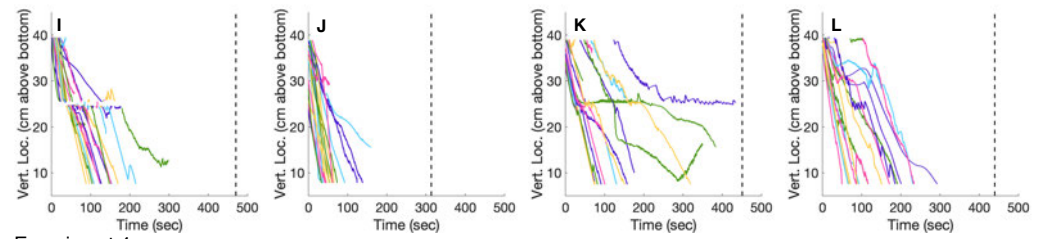
Experiment 1



Experiment 2



Experiment 3



Experiment 4

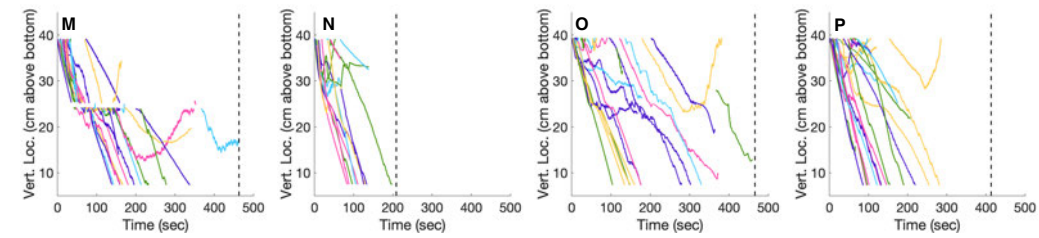


Figure 6. Vertical distributions combined for all four experiments showing number of copepods (shown in color bar) in discrete depth bins vs. time for each treatment: (A) the layer treatment, (B) the homogenous treatment, (C) the control with a gradient treatment, and (D) the control with no gradient treatment.

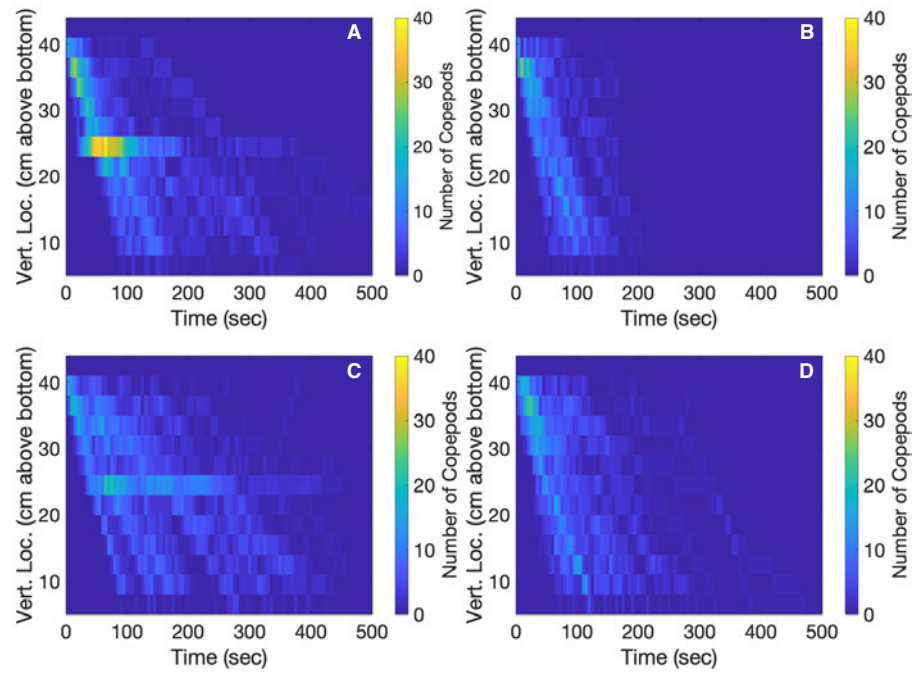
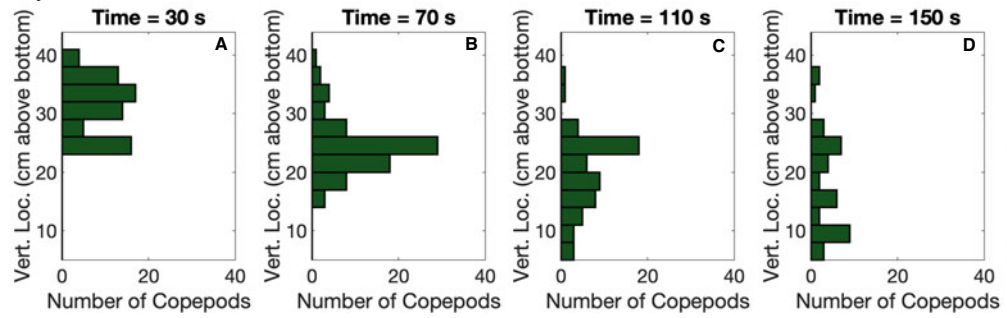
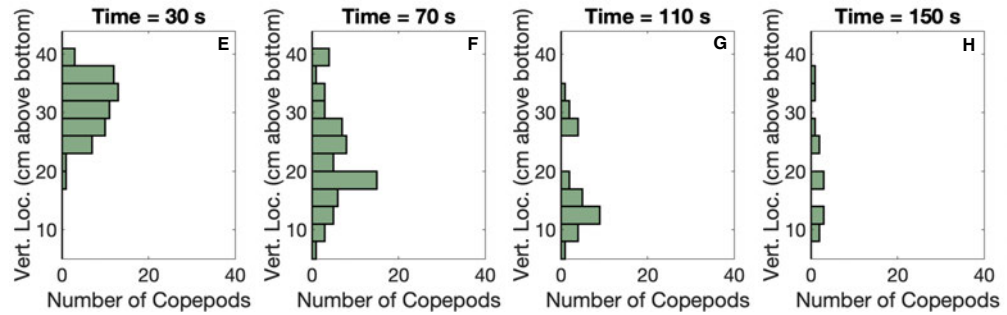


Figure 7. Histograms showing copepod vertical distribution at four time points (30, 70, 110, and 150 seconds) in the layer treatment (A-D), homogenous treatment (E-H), control with a gradient treatment (I-L), and control with no gradient treatment (M-P).

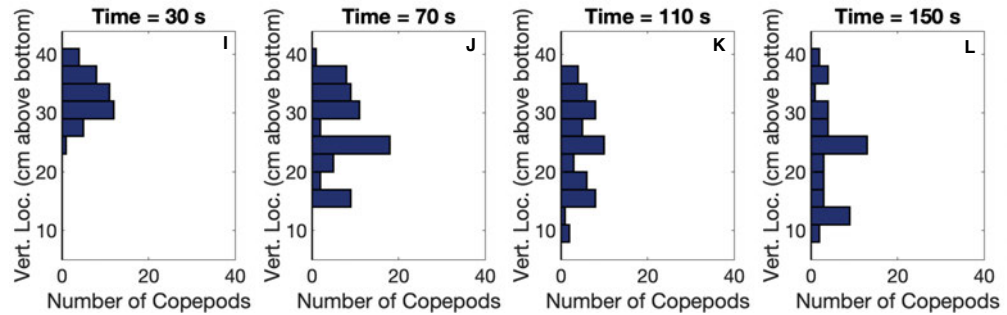
Layer



Homogenous



Control with Gradient



Control with No Gradient

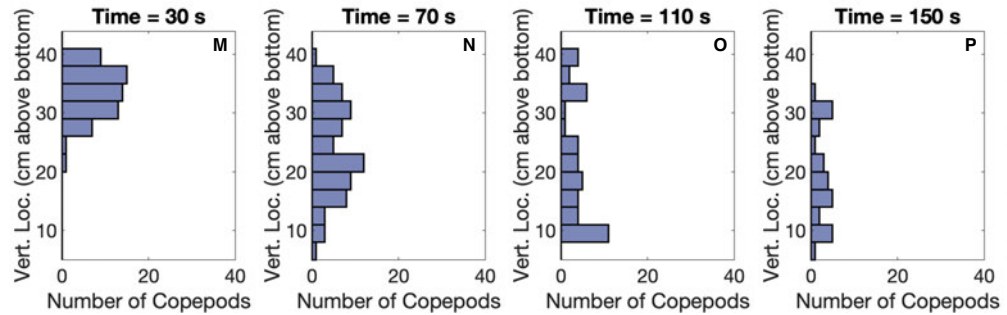


Figure 8. Bar graph showing average residence time in the layer region (defined in Methods) between treatments. Error bars represent standard error across experiments (n=4). One-way ANOVA demonstrated significant difference between treatments ($P<0.001$). Treatments not sharing orange letters represent significant differences based on Tukey–Kramer *post hoc* test.

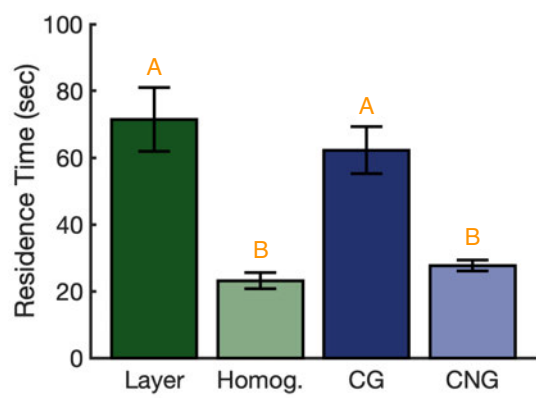


Figure 8. Bar graphs comparing average values of behavioral properties (A, B: overall velocity; C, D: vertical velocity, with positive values representing downward velocities; E, F: jump frequency; G, H: NGDR, and I, J: vertical NGDR) between treatments for the whole tank (left panels) and only within the layer region (right panels). Error bars represent standard error. One-way ANOVA showed significant difference between treatments for (A, $P=0.030$) (B, $P<0.001$), (C, $P<0.001$), (D, $P<0.001$), (E, $P=0.020$), (F, $P<0.001$), (H, $P<0.001$), (I, $P<0.001$), and (J, $P<0.001$), but no significant difference between treatments for (G, $P=0.200$). Treatments not sharing orange letters represent significant differences based on Tukey–Kramer *post hoc* test.

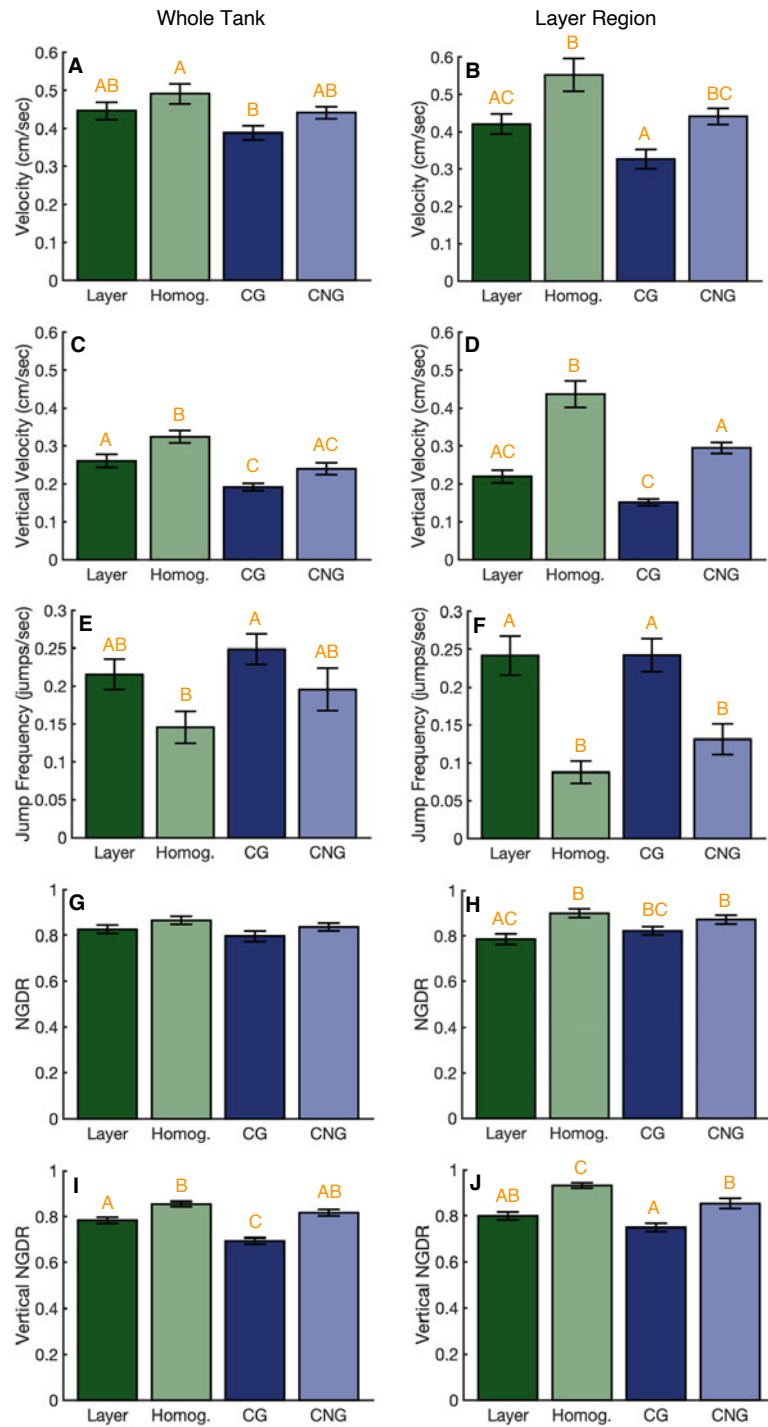
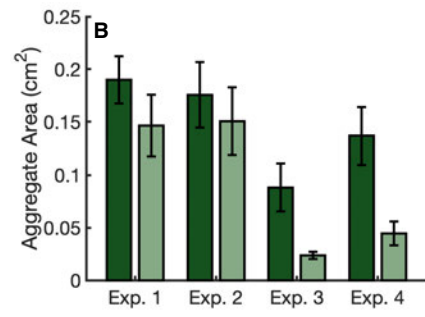
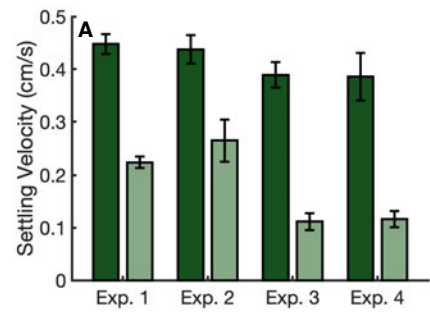


Figure 10. Bar graphs showing average (A) aggregate settling velocity and (B) aggregate area between layer (dark green) and homogenous (light green) treatments across all four experiments. Error bars represent standard error.



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CHAPTER 4: Conclusion

This study showed that the distribution of marine snow does impact the foraging behavior of *Calanus pacificus*. Copepods did ingest marine snow aggregates when exposed to both the layer and homogenous distributions of marine snow, confirming active consumption of marine snow by *tyuiop-5r4e* as observed in previous studies (Dilling *et al.* 1998, Cawley *et al.* 2021). In addition, ingestion of aggregates was potentially higher in the layer distribution than in the homogenous distribution.

We also observed accumulations of copepods at the center of the tank in the two treatments containing a density gradient (layer and control with gradient), with no such accumulation observed in the two treatments without a density gradient (homogenous and control with no gradient).

Differences in swimming properties were observed across treatments, particularly when analyzing differences in behavior specifically within the layer region of our tanks. Here, the results demonstrated that jump frequency was higher and vertical velocity was lower in the layer treatment and control with gradient treatment compared to the two treatments with no density gradient. This behavior is consistent with the finding that residence time was higher within the layer region of these two treatments, as increased jump frequency and decreased vertical velocity allowed the copepods to remain in the layer region for longer periods of time. Such behavior is also consistent with findings from a previous study by Tiselius (1992), where copepods that were exposed to a layer of phytoplankton altered their behavior in order to exploit this patch of food. Our

study shows that copepods are able to alter their behavior to locate patches of food made up of marine snow aggregates, and since sinking marine snow commonly forms thin layers at density discontinuities (McIntyre *et al.* 1995, Alldredge *et al.* 2002, McManus *et al.* 2003, Prairie *et al.* 2010), understanding that they are able to alter their foraging behavior to locate and remain in these food patches is crucial.

Interestingly, while we did see differences in behavior between the two treatments with a density gradient and the two treatments without a density gradient, we generally did not see differences in behavior or residence time between copepods in the layer treatment and the control with gradient treatment. This makes it difficult to determine whether individuals are reacting to the presence of the salinity gradient or the presence of a food patch. That being said, copepods did display a lower path linearity (lower NGDR) in the layer treatment (with this difference being significant compared to the two treatments without the density gradient), suggesting that path linearity may have decreased directly in response to the presence of food at the layer. The experimental study by Tiselius (1992) also had controls for the experiments in which copepods were exposed to a sharp change in salinity (halocline) without a phytoplankton thin layer present. In these controls, it was observed that copepods frequently made loops and temporarily increased their velocities when exposed to the halocline and suggested that the individuals may have been searching for the presence of food, with the sharp change in salinity acting as a physical cue for the presence of a potential food patch (Tiselius 1992). A similar pattern is evident in our study,

where copepods in the control with gradient treatment had a lower vertical path linearity in the layer region than copepods in the layer treatment. These results suggest that, without the food signal in the control with gradient treatment, the copepods exposed only to a density gradient and no food continued to search in a way that the copepods that were exposed to a density gradient and a food patch did not (Woodson *et al.* 2007).

To explain this behavior, we propose a cue hierarchy, where the physical change in salinity may be acting as a primary cue to limit search regions and then a secondary chemical cue created by the presence of food elicits a secondary response, as was proposed in a previous study by Woodson *et al.* (2007). While we did not observe significant differences in residence time in the layer region between the layer treatment and control with gradient treatment, copepod vertical distribution over time did show some subtle but noteworthy differences between the two treatments. We observed a stronger initial accumulation of copepods at the layer depth in the layer treatment, but most individuals in the layer treatment left the layer region by the 150-second time point, whereas several copepods in the control with a gradient treatment still remained within the layer region at 150 seconds, suggesting that accumulation of copepods in the presence of density gradient may be weaker but last longer when no food is present. However, because of limitations in our experimental design, we could not track copepods when they were located within the layer of aggregates in the layer treatment.

While other experimental studies have shown that copepods increase their residence time and alter their swimming properties in phytoplankton layers

(Tiselius 1992, Menden-Deuer and Grünbaum 2006), this is the first study to track individual behavior of copepods through a marine snow thin layer in comparison to a homogenous distribution of marine snow. It is critical to understand whether copepods can alter their foraging behavior to utilize patchy distributions of marine snow in order to make more accurate estimates of copepod distributions and grazing rates. Furthermore, the spatial heterogeneity in zooplankton populations caused by their behavioral responses to marine snow layers has important implications for organisms at higher trophic levels, and thus pelagic ecosystems as a whole (Mackas *et al.* 1985, Pinel-Alloul 1995).

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APPENDIX

APPENDIX A. Bar graphs showing average raw gut pigment content between all four treatments for (A) Experiment 1, (B) Experiment 2, (C) Experiment 3, and (D) Experiment 4, where CG = control with a gradient and CNG = control with no gradient. Error bars represent standard error. One-way ANOVA demonstrated significant difference between treatments in all four experiments (A: $P=0.009$, B: $P<0.001$, C: $P=0.001$, D: $P=0.008$). Treatments not sharing orange letters represent significant differences based on Tukey–Kramer *post hoc* test.

