PHYSIOLOGICAL MECHANISMS FACILITATING MORPHOLOGICAL PLASTICITY ACROSS HYDRODYNAMIC GRADIENTS IN KELPS

by

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Abstract

Some kelp species exhibit morphological plasticity across gradients of hydrodynamic forcing. Such kelps tend to grow narrow, flat blades in fast flow and broad, undulate blades in slow flow. This plasticity is an adaptive phenomenon that allows the kelps that show it to continuously reduce drag while enhancing productivity. While the functional consequences of this phenomenon have been relatively well studied, the developmental mechanisms that underlie it are poorly understood. The primary goal of this thesis is to improve our understanding of the developmental processes facilitating morphological plasticity across hydrodynamic gradients in kelps. I first conducted several experiments where I applied tension to blade tissue of the kelp *Nereocystis luetkeana* in various ways to better characterize the growth response to mechanical stimulation normally imposed by drag. The results of these experiments suggest that plasticity in kelps is probably regulated at the scale of individually stimulated cells and that changes in blade morphology are likely brought about through changes in the direction of cell growth and/or division (Ch. 2). I then examined the effects of auxin on *Nereocystis* blade growth and morphology and considered whether auxin could play a role in mediating kelp plasticity. I found that auxin can have morphogenic effects in *Nereocystis* blade tissue that are remarkably similar to the effects of tension (Ch. 3). Next, I tested whether culturing the kelp *Macrocystis pyrifera* in reduced concentrations of Ca^{2+} could inhibit the growth response to mechanical loading, which would suggest that Ca^{2+} signaling might play a role in regulating plasticity. No evidence arose that reducing the ambient Ca^{2+} concentration could inhibit plasticity (Ch. 4). Furthermore, all Ca^{2+} reductions greater than 50% proved lethal for kelps (Ch. 4). Finally, I investigated how prevalent phenotypic plasticity across hydrodynamic gradients is in various algal groups and considered whether mechanisms of flow perception could limit the evolution of such plasticity. I

found that (1) researchers examining intraspecific variation across flow gradients in seaweeds usually have not tested for plasticity and (2) verified plasticity has been documented more frequently in brown algae with intercalary growth than it has in other macroalgae (Ch. 5).

Lay Summary

Some kelps can change their shape based on the amount of water motion in their environment. This is an adaptation that allows these kelps to reduce the risk of mortality caused by fast flow speeds while increasing productivity. While research has been conducted examining the functional consequences of this phenomenon, little is known about the developmental mechanisms that underlie it. In this thesis, I endeavour to improve our understanding of the developmental mechanisms that facilitate kelp morphological plasticity across gradients of water motion. I first conducted several experiments to better characterize the kelp growth response to tension normally imposed by flow. I also explored whether several signaling mechanisms known from plants are also involved in regulating plasticity in kelps. Finally, I investigated how prevalent plasticity across flow gradients is across all seaweeds and considered whether mechanisms of flow perception might constrain the evolution of this trait in some seaweed groups.

Preface

Chapter 2: I designed the experiments described in this chapter with input from P. Martone. I collected the data with some assistance from A. Breitkreutz. I performed all data analysis and visualization with input from P. Martone. I wrote the manuscript with input from P. Martone. A version of this chapter is published as:

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Chapter 3: I designed the experiment described in this chapter with input from P. Martone and S. Singh. I collected all data. I performed all data analysis and visualization with input from P. Martone.

Chapter 4: I designed the experiments described in this chapter with input from P. Martone and A. Rosado. I collected all data. I performed all data analysis and visualization with input from P. Martone.

Chapter 5: I designed the methods described in this chapter with input from P. Martone. I collected all data and performed all data analysis and visualization with input from P. Martone.

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List of Abbreviations

A	Area of an object
A_B	Blade area
A_C	Mean meristoderm cell area
A_S	Kelp tissue sample area
BMSC	Bamfield Marine Sciences Centre
C_D	Drag coefficient
DBL	Diffusive boundary layer
F_D	Force of drag
IAA	Indole-3-acetic acid
L_B	Blade length
L_C	Mean meristoderm cell length
L_P	Projected length of blade edge
L_S	Kelp tissue sample length
L_T	Total length of blade edge
M_B	Blade wet mass
M_S	Kelp tissue sample wet mass
NAA	1-napthaleneacetic acid
PCA	Principal components analysis
PC1	First principal component
PC2	Second principal component
PC3	Third principal component
PC4	Fourth principal component

R	Blade ruffle
SMA	Standard major axis
T	Blade thickness at 10 cm from the origin
U	Fluid velocity
UBC	University of British Columbia
W_B	Blade width at 10 cm from the origin
W_C	Mean meristoderm cell width
W_S	Kelp tissue sample width
ΔA_B	Change in blade area
ΔA_S	Change in kelp tissue sample surface area
ΔL_B	Change in blade length
ΔL_D	Change in disk length
ΔL_S	Change in kelp tissue sample length
ΔM_B	Change in blade wet mass
ΔM_D	Change in disk wet mass
ΔM_S	Change in kelp tissue sample wet mass
ΔR	Change in blade ruffle
ΔT	Change in blade thickness at 10 cm from the origin
ΔW_B	Change in blade width at 10 cm from the origin
ΔW_D	Change in disk width
ΔW_S	Change in kelp tissue sample width
ρ	Fluid density

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I need to acknowledge the Bamfield Marine Sciences Centre and its staff for the instrumental role they played in my winding up where I am today. Field trips I took to BMSC as a high school student and undergraduate provided early inspiration that led me to pursue further study of marine science. This culminated in my participating in the 2013 Fall Program, which not only solidified my love of seaweed, but also facilitated me meeting Patrick Martone and, by extension, becoming a graduate student. Later, BMSC provided me with facilities for conducting several experiments, including one of the experiments discussed in Chapter 2 of this thesis. I would like to extend my sincerest thanks to the staff of BMSC for helping make all of this happen.

Finally, I would like to thank my family for always supporting and encouraging me throughout my time as a graduate student. I would also like to thank my wonderful partner Tatiana for her endless support and for keeping me alive while I wrote my thesis.

Dedication

This thesis is dedicated to my parents, who always encouraged me to follow my dreams and never failed to support me along the way.

1. Introduction

"…the seed of creation is the strife of organisms at odds with their changing environments."

-Thomas J. DeWitt & R. Brian Langerhans

1.1 The evolution of phenotypic plasticity

Phenotypic plasticity is the environmentally-mediated expression of variable phenotypes by a single genotype (Bradshaw 1965, Stearns 1989). This phenotypic variation can be the direct result of environmental influence (e.g. variation in metabolic rates with temperature) or it can be actively facilitated by the organism (e.g. development of defensive spines in response to chemical cues from a predator; Smith-Gill 1983, Stearns 1989). Plasticity is ubiquitous in nature (Travis 1994) and can occur in a highly diverse range of traits, such as morphology (e.g. Cook and Johnson 1968, Harvell 1984, Gerard 1987), physiology (e.g. Zangerl and Bazzaz 1983, Ragazzola et al. 2013), and life history (e.g. Lotz and Blom 1986, Reznick 1990, Nussey et al. 2005). It can be facilitated by an equally great diversity of environmental factors, including but not limited to temperature (e.g. Zangerl and Bazzaz 1983, Kübler and Dudgeon 1996, Morin et al. 1997), light (e.g. Dring and Lüning 1975, Pigliucci et al. 1995), chemicals (e.g. Palmer 1990), and mechanical forces (e.g. Gerard 1987, Coutand et al. 2000). Even though phenotypes generated through plasticity are not generally heritable, phenotypic plasticity itself is a trait of genetic origin that can be acted on by selection and evolve (e.g. Gotthard and Nylin 1995, DeWitt and Scheiner 2004). Furthermore, a trait and plasticity of that trait can be considered separate characters that are capable of evolving independently of one another (e.g. Via and Lande 1985, Via et al. 1995, De Jong 2005).

Phenotypic plasticity can have a positive, neutral, or negative effect on organism fitness (Stearns 1989, Relyea 2002, Ghalambor et al. 2007). Plasticity that increases fitness is termed adaptive plasticity (Stearns 1989, Ghalambor et al. 2007). For purposes of this thesis, I will collectively use the term "non-adaptive plasticity" to refer to plasticity that has a neutral or negative effect on fitness (Ghalambor et al. 2007). Historically, there has been a great deal of research interest in the phenomenon of adaptive plasticity, which is generally viewed as an important evolutionary mechanism for coping with spatial or temporal environmental heterogeneity. Indeed, having a flexible phenotype can, in some circumstances, permit a single genotype to tolerate a broader range of conditions and maintain higher fitness across multiple environments than it would be able to with any fixed phenotype (e.g. Bradshaw 1965, Schlichting and Pigliucci 1998, Pigliucci 2001). However, recent research suggests that most plasticity is actually non-adaptive (De Jong 2005, Van Kleunen and Fischer 2005, Ghalambor et al. 2007) and there remain specific situations in which fixed phenotypes are predicted to be more selectively favourable than flexible ones (e.g. Via and Lande 1985, Van Tienderen 1997). A commonly proposed explanation for why adaptive plasticity is not more prevalent in nature is that plasticity comes with inherent costs and limits that can constrain its evolution (e.g. DeWitt et al. 1998, Relyea 2002, Auld et al. 2010, Murren et al. 2015). Potential costs of plasticity might include energetic expenses of sensing relevant environmental cues or developmentally implementing phenotypic changes, while limits might include the reliability of environmental cues and the length of time required to develop a phenotypic response (DeWitt et al. 1998).

Given that phenotypic plasticity can be adaptive in nature but also that its evolution can be constrained, when would it be expected to be favoured over the development of fixed phenotypes? At the most basic level, evolutionary models predict that, given genetic variation,

adaptive plasticity will evolve when: (1) populations are subject to environmental heterogeneity; (2) specific environments are associated with reliable cues; (3) different environments selectively favour different phenotypes; and (4) superior fitness cannot be achieved across all environments with any single fixed phenotype (DeWitt and Scheiner 2004, Ghalambor et al. 2007). However, fitness costs may affect how generally these predictions may be applied (DeWitt et al. 1998, Relyea 2002, Ghalambor et al. 2007). For example, plasticity is likely to only be a favourable strategy for organisms subjected to temporal environmental heterogeneity when the response time for detecting and responding to an environmental change is short relative to the duration of the new set of conditions (Padilla and Adolph 1996, DeWitt et al. 1998, Alpert and Simms 2002). The model predictions described above appear to be supported by the observations that early successional and annual plant species appear more likely to exhibit a high degree of plasticity than late successional and perennial species (Cook and Johnson 1968, Wilken 1977, Zangerl and Bazzaz 1983).

1.2 General biology of kelps

Kelps are brown algae belonging to the order Laminariales (Graham et al. 2017). This charismatic group of seaweeds includes the largest and most structurally complex of the world's algae (Fritsch 1923). Kelps have heteromorphic sporic life histories that include a microscopic, filamentous gametophyte stage and a macroscopic sporophyte stage constructed of three-dimensionally growing parenchyma (Graham et al. 2017). This complex tissue construction is key to the ability of species such as *Macrocystis pyrifera* to reach lengths of over 50 m (Graham et al. 2017). The kelp body plan outwardly resembles that of a land plant and consists of a root-

like holdfast, usually a stem-like stipe, one or more leaf-like blades, and sometimes one or more gas-filled pneumatocysts (Druehl and Clarkston 2016, Graham et al. 2017; Fig. 1.1). The holdfast serves to anchor the kelp to the substratum; unlike the roots of land plants, it plays no role in water or nutrient acquisition (Graham et al. 2017). The stipe functions mostly as structural support and blades are the primary photosynthetic organs (Graham et al. 2017). Pneumatocysts provide buoyancy and help increase light interception by lifting blades closer to the water's surface (Graham et al. 2017, Liggan and Martone 2018). Growth in kelps occurs primarily through the activity of an intercalary meristem located at the junction(s) between the blade(s) and stipe (Fritsch 1923), which results in blade tissue being gradually shuttled distally like a conveyor belt as new growth progresses (Kain 1987, Koehl et al. 2008). Kelps such as *Macrocystis pyrifera* and *Nereocystis luetkeana* are some of the fastest growing organisms in the world, with the blades of *Nereocystis* in particular having been observed to elongate at rates as high as 14 cm day^{-1} (Kain 1987).

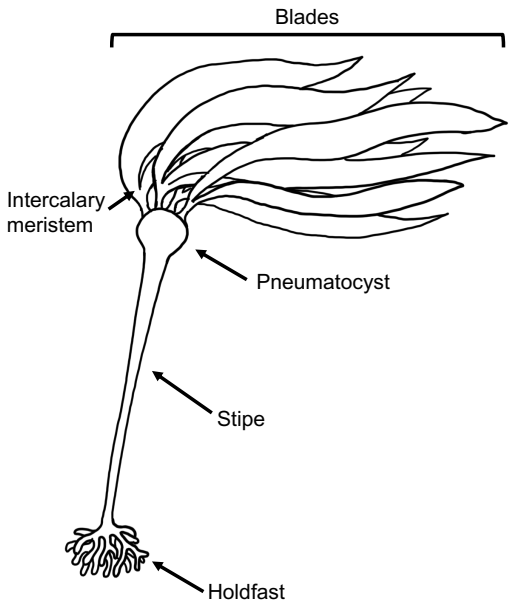

Fig. 1.1. General body plan of the kelp *Nereocystis luetkeana*.

Kelp thalli are composed of three tissue layers. The outermost layer, termed the meristoderm, is the only pigmented layer and is the only layer in which cell division occurs (Fritsch 1923). The cells of this layer are approximately isodiametric and are smaller than the cells of the other layers (Fritsch 1923). Beneath the meristoderm lies the cortex, which is a relatively thick layer consisting of colourless cuboid cells that tend to increase in size the further below the meristoderm they are found (Fritsch 1923). Cortex cells do not divide but enlarge over time to keep up with the rapidly dividing cells of the meristoderm layer (Fritsch 1923). The innermost tissue layer is the medulla, a layer of elongate, filamentous cells that, similarly to cortex cells, do not divide but elongate over time (Fritsch 1923). This layer contains sieve elements that are capable of translocating photosynthate throughout the body of the kelp, much like the phloem of land plants (Nicholson and Briggs 1972, Graham et al. 2017). These structures and the abilities they grant are unique in the algal world and contribute to the ability of kelps to reach such large sizes (Schmitz and Lobban 1976, Graham et al. 2017).

Brown algal cell walls are mostly composed of alginate, but, like those of land plants, they also contain cellulose microfibrils (Kloareg and Quatrano 1988, Graham et al. 2017). Even though cellulose generally only makes up 1-10% of brown algal body dry mass (Graham et al. 2017), it is still the primary load-bearing component of the brown algal cell wall (Kloareg and Quatrano 1988). Unlike land plants, brown algae lack cortical microtubules (Katsaros et al. 2006) and cellulose microfibril patterning in brown algal cell walls is controlled by actin filaments (Katsaros et al. 2002, 2006). Brown algal cells, unlike those of some other algae (e.g. Pueschel 1977), have plasmodesmata (Terauchi et al. 2015).

In addition to being important primary producers in temperate nearshore marine ecosystems (Mann 1973, Brady-Champbell et al. 1984, Reed et al. 2008, Krumhansl and Scheibling 2012), their large size allows kelps to form three-dimensional "forests" that provide habitat and nurseries for a wide diversity of organisms (Steneck et al. 2002, Graham 2004, Siddon et al. 2008, Teagle et al. 2017, Miller et al. 2018). These forests also provide ecosystem services to humans that can rival those of coral reefs in terms of economic value (Bennett et al. 2016). Ecosystem services provided by kelp forests include facilitating production of economically significant fish and invertebrate species (Graham 2004, Shaffer 2004, Bennett et al. 2016, Teagle et al. 2017) and promoting tourism (Bennett et al. 2016). Similarly to terrestrial forests (e.g. Mestre et al. 2017, Keren 2020), kelp forests can be described in terms of vertical layers with a canopy at the top and an understory near the benthos (Steneck et al. 2002, Clark et al. 2004, R. J. Miller et al. 2011). Kelp forest canopies are typically composed of either large buoyant species that float near the surface, such as *Macrocystis* or *Nereocystis*, or larger stipitate kelps, such as *Pterygophora* or *Ecklonia*, that remain suspended above the benthos by stiff stipes (Steneck et al. 2002). Some canopy-forming kelps, such as *Nereocystis*, have annual life cycles, which results in some entire forest canopies dying off and re-growing to full height every year (Rigg 1912, Foreman 1984, Steneck et al. 2002).

In spite of the many anatomical and ecological similarities between kelps and land plants (Steneck et al. 2002, Drobnitch et al. 2015, Druehl and Clarkston 2016, Starko and Martone 2016a) the two groups are distantly related within the tree of eukaryotes (Keeling and Burki 2019). Apparent commonalities between them are generally the result of convergent evolution (e.g. Drobnitch et al. 2015, Starko and Martone 2016a). However, some plant metabolic pathways, including those of photosynthesis and cellulose synthesis, have been acquired by kelps

horizontally through the endosymbiotic assimilation of a red alga that gave rise to the brown algae (Keeling 2004, Cock et al. 2010, Michel et al. 2010). This has led to kelps and plants having numerous cellular features in common.

1.3 Biomechanical adaptations to hydrodynamic forcing in seaweeds

Water motion poses a complex evolutionary challenge for seaweeds. On one hand, exposure to at least a moderate degree of water movement is often beneficial for primary producers like marine macroalgae (e.g. Wheeler 1980, Gerard 1982, Hurd et al. 1996). The diffusive boundary layer (DBL) is a thin layer of water that forms near the surface of a seaweed within which diffusion in and out of the algal tissue is the dominant mechanism driving the movement of dissolved material (Gundersen and Jorgensen 1990, Hurd 2000). The thickness of this layer is proportional to the velocity of the surrounding water (Hurd 2000). When ambient flow speeds are sufficiently slow, the DBL thickens to a point where dissolved nutrients within it can be depleted, which can reduce productivity and growth in macroalgae (e.g. Wheeler 1980, Gerard 1982, Hurd et al. 1996). This can make it advantageous for algae to live in environments with more water movement (Gerard and Mann 1979, Leigh et al. 1987, Hurd 2000, 2017).

On the other hand, while some water movement helps increase productivity in seaweeds (e.g. Wheeler 1980, Gerard 1982, Hurd et al. 1996), excessively high amounts of motion can become hazardous. In wave-exposed intertidal zones, for instance, water velocities of 2-3 m s^{-1} are commonplace (Carrington Bell and Denny 1994, Denny and Gaylord 2002, Denny et al. 2003) and speeds of up to 25 m s^{-1} have been recorded (Denny and Gaylord 2002). Water traveling at such speeds imposes tremendous amounts of force on objects it flows past and only organisms that have evolved ways of avoiding wave-induced breakage or dislodgement will be

able to survive in environments subject to this level of water motion (Koehl 1984, Denny et al. 1985, Denny and Gaylord 2002, Denny 2006).

The force imposed on organisms by moving water is primarily oriented in the direction of flow, and is therefore defined as drag (Vogel 1984, Gaylord 2000). The drag experienced by an object in flow can be calculated as follows:

$$F_D = \frac{1}{2}\rho U^2 A C_D$$

where F_D is the force of drag (N), ρ is the density of the fluid (kg m^{-3}), U is the velocity of the fluid relative to the object (m s^{-1}), A is the projected area of the object (m^2), and C_D is the drag coefficient, a dimensionless index associated with the shape of the object (Vogel 1996). If a seaweed is to survive in an environment with fast flow conditions, it must prevent F_D from reaching a magnitude where the seaweed's support tissues are broken or the thallus is dislodged from the substratum. Marine macroalgae have evolved a myriad of solutions to this problem, which can largely be categorized as either drag avoidance strategies, which are based around decreasing the value of F_D experienced by the algal thallus, or drag tolerance strategies, which are based on increasing the magnitude of F_D necessary to break or dislodge the alga (Starko and Martone 2016b). Common drag avoidance strategies in seaweeds include reducing the amount of drag experienced by limiting thallus size (Carrington 1990, Blanchette 1997, Wolcott 2007), adopting streamlined morphologies (Koehl and Alberte 1988, Armstrong 1989, Haring and Carpenter 2007), and flexibly reconfiguring in flow (Boller and Carrington 2006, Martone et al. 2012). Large kelps like *Nereocystis* can also use the flexibility of their tissues to "go with the flow" as waves roll past them, which keeps their stipes slack and potentially allows them to avoid mechanical loads from being imposed on their support tissues at all (Koehl 1984, Friedland and Denny 1995). Drag tolerance strategies in seaweeds include increasing the

strength of attachment to the substratum (Milligan and DeWreede 2000, Starko et al. 2014) and increasing the amount of energy required to break support tissues by making them stronger (Milligan and DeWreede 2000, Martone 2006, 2007) or more extensible (Koehl and Wainwright 1977, Holbrook et al. 1991).

1.4 Morphological plasticity across hydrodynamic gradients in kelps

Some species of kelps show conspicuous variability in the morphology of their blades across gradients of hydrodynamic forcing. Species such as *Nereocystis luetkeana*, *Macrocystis pyrifera*, and *Saccharina latissima*, which are all known to grow in a range of hydrodynamic conditions, develop broad and sometimes undulate blades when growing in wave- and current-sheltered environments, but narrow and flat blades when growing in hydrodynamically stressful environments (Druehl 1978, Koehl and Alberte 1988, Buck and Buchholz 2005; Fig. 1.2). Narrow blades experience less drag in flow, while broad blades are better at intercepting light (Koehl and Alberte 1988, Johnson and Koehl 1994, Buck and Buchholz 2005). It has been demonstrated through transplantations and similar manipulative experiments that these variations in morphology are the result of phenotypic plasticity (Druehl and Kemp 1982, Buck and Buchholz 2005, Koehl et al. 2008). This plasticity has generally been interpreted as an adaptive phenomenon that permits kelps like *Nereocystis* to continuously maintain the most optimal blade morphology for a given flow environment (Koehl and Alberte 1988, Koehl et al. 2008).

Most research that has examined kelp morphological plasticity has focused on its functional significance (e.g. Koehl and Alberte 1988, Johnson and Koehl 1994, Buck and Buchholz 2005, Hurd and Pilditch 2011). In comparison, little is known about what developmental mechanisms allow morphologically plastic kelp species to alter their blade shapes

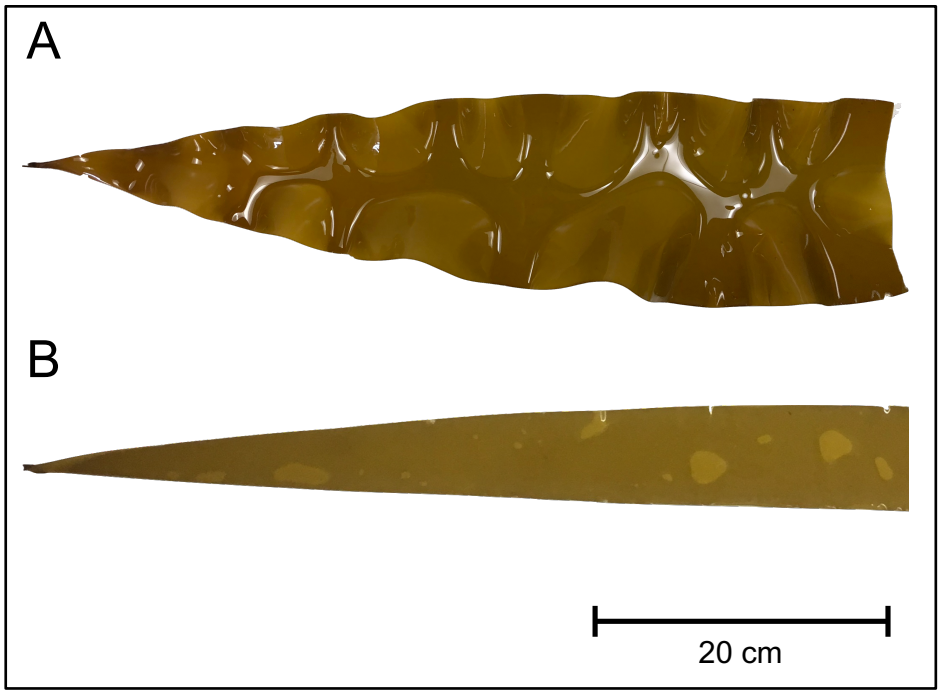

Fig. 1.2. ***Nereocystis luetkeana*** **blades grown in (A) a wave-sheltered environment and (B) a wave-swept environment.**

in response to changes in the hydrodynamic environment. Experiments in which the effect of drag was simulated using hanging weights have shown that the plasticity is a response to mechanical stimulation (Gerard 1987, Koehl et al. 2008). Gerard (1987) speculated, but did not clearly demonstrate, that the observed narrowing of *Saccharina latissima* blades when tensile stress was applied was the result of the kelp reorienting the direction of cell divisions. Additionally, Koehl et al. (2008) concluded that the undulations that developed in the blades of *Nereocystis* when the kelp grew in sheltered environments were due to blade margins elongating more quickly than blade midlines. Overall, the exact changes in growth patterns that result in the development of different blade morphologies need to be clarified, and mechanisms of mechanoperception and signal transduction that might facilitate the translation mechanical cues into those changes in growth patterns remain entirely unknown.

1.5 Thesis overview

The goal of this thesis is to improve our understanding of the developmental mechanisms facilitating morphological plasticity across hydrodynamic gradients in kelps. The research described herein was mostly conducted in the bull kelp, *Nereocystis luetkeana*, and draws a great deal of inspiration from the land plant literature for purposes of formulating hypotheses. I first endeavour to produce a more detailed picture of how mechanical loading affects kelp growth. I then examine whether two specific mechanoperception and signal transduction mechanisms known to occur in plants might also play a role in facilitating kelp morphological plasticity. Finally, I examine how prevalent phenotypic plasticity across hydrodynamic gradients really is in seaweeds and consider whether the mechanistic bases of this trait could potentially constrain its evolution in some algal groups.

In Chapter 2, I explore how growth of *Nereocystis* blades is affected when tensile stimulation is applied in varying amounts, directions, and locations. I then consider what my observations might reveal about the developmental mechanisms underlying kelp morphological plasticity, as well as the evolutionary significance of this phenomenon for the kelp. I show that *Nereocystis* blades can fine-tune their shape according to an applied force, growing continuously longer and narrower with increasing magnitude of applied tensile force. I show that changing the direction of the tensile stimulus will change the growth response, and that the morphology of a blade is reflected in the morphology of its cells. Finally, I show that applying a mechanical load to the distal, non-growing tissue of a blade does not evoke a morphological response in that blade's growing tissue. Collectively, these observations indicate that (1) *Nereocystis* is well adapted to a broad range of hydrodynamic environments, (2) the developmental process underlying morphological plasticity in *Nereocystis* is likely very localized to individually

stimulated cells and probably does not involve long-distance signaling, and (3) changes in blade morphology induced by mechanical loading may result from changes in the direction of meristoderm cell growth and/or division.

In Chapter 3, I examine whether the application of the plant hormone auxin at various concentrations can induce growth or morphogenic changes in *Nereocystis* blade tissue, and I consider whether auxin signaling could play a role in mediating the observed effects of mechanical loading on kelp growth. I show that a 10^{-5} M auxin treatment causes *Nereocystis* blade tissue to grow longer and narrower, but not heavier, compared to a control group, indicating that auxin can have morphogenic effects on *Nereocystis* without affecting overall growth. While this is not evidence that auxin is involved in mediating kelp morphological plasticity in response to mechanical loading, the observed morphological effects of auxin application and mechanical stimulation are remarkably similar and further study is recommended.

In Chapter 4, I investigate if reducing the ambient Ca^{2+} concentration can disrupt the response of young sporophytes of the kelp *Macrocystis pyrifera* to mechanical loading. If so, this would suggest that plasma membrane-bound stretch activated Ca^{2+} channels might be involved in the process by which kelps perceive the mechanical cues that mediate plasticity across hydrodynamic gradients. I show that reducing the ambient Ca^{2+} concentration by 50% has no effect on the response of *Macrocystis* to mechanical loading, which may indicate that (1) stretch activated Ca^{2+} channels do not play a role in the kelp's mechanoperception mechanism, (2) stretch activated channels are involved in mechanoperception, but a 50% reduction in Ca^{2+} concentration is not a large enough reduction to interfere with the process, or (3) Ca^{2+} signaling is involved in mechanoperception, but the ions come from internal cellular stores and the process

does not rely on plasma-membrane bound channels. I also show that any reduction in Ca^{2+} concentration of more than 50% is lethal for kelps.

In Chapter 5, I conduct a review of the literature to find out how often phenotypic plasticity across hydrodynamic gradients has been identified in different groups of seaweeds. I consider biomechanical and developmental mechanisms that might be required for such plasticity and then examine whether the tendency for this plasticity to arise in a seaweed might depend on that seaweed's growth mode and flow environment. I show that, while researchers have often observed instances of intraspecific phenotypic variation across hydrodynamic gradients in seaweeds, they have only explicitly tested whether such variation is due to plasticity 35% of the time. I also show that phenotypic plasticity across hydrodynamic gradients has been documented considerably more often in brown algae, specifically those with intercalary meristems, than in red or green algae, which do not exhibit intercalary growth. This may suggest that intercalary meristems help facilitate the evolution of phenotypic plasticity across hydrodynamic gradients in seaweeds by acting as a platform for sensing flow.

2. Morphological plasticity in the kelp *Nereocystis luetkeana* (Phaeophyceae) is sensitive to the magnitude, direction, and location of mechanical loading

2.1 Synopsis

Nereocystis luetkeana is a canopy-forming kelp that exhibits morphological plasticity across hydrodynamic gradients, producing broad, undulate blades in slow flow and narrow, flattened blades in fast flow, enabling thalli to reduce drag while optimizing photosynthesis. While this phenomenon has been relatively well studied from a functional perspective, the developmental and physiological mechanisms that facilitate the plasticity remain poorly understood. In this study, we conducted three experiments to characterize how the (1) magnitude, (2) direction, and (3) location of plasticity-inducing mechanical stimuli affect the morphology of *Nereocystis* blades. We found that applying a gradient of tension caused blades to grow progressively longer, narrower, less ruffled, and heavier in a linear fashion, suggesting that *Nereocystis* is equally well adapted for all conditions within its hydrodynamic niche. We also found that applying tension transversely across blades caused the growth response to rotate 90°, indicating that there is no substantial separation between the sites of stimulus perception and response. This also suggests that a long-distance signaling mechanism, such as a hormone, is unlikely to mediate this phenomenon. Meristoderm cells showed morphological changes that paralleled those of their respective blades in this experiment, implying that tissue-level morphology is influenced by cell growth. Finally, we found that plasticity was only induced when tension was applied directly to the growing tissue, reinforcing that long-distance signaling

is probably not involved and possibly indicating that the mechanism on display generally requires an intercalary meristem to facilitate mechanoperception.

2.2 Introduction

Moving water presents marine macroalgae with a complex evolutionary challenge. While high levels of water motion can be beneficial to these organisms by improving mass transfer rates across diffusion boundary layers and increasing primary productivity (Wheeler 1980, Gerard 1982, Hurd et al. 1996), excessively high levels can cause attachment or support tissues to fail, generally resulting in mortality (Koehl and Wainwright 1977, Blanchette 1997, Duggins et al. 2001, Demes et al. 2013). To reap the benefits of flow while mitigating its hazards, seaweeds have adopted a diverse range of biomechanical and evolutionary strategies (Koehl and Wainwright 1977, Denny and Gaylord 2002, Martone et al. 2012, Starko and Martone 2016b).

Nereocystis luetkeana (hereafter referred to as *Nereocystis*) is a canopy-forming annual kelp that grows in a broad range of hydrodynamic environments (Abbott and Hollenberg 1976, Johnson and Koehl 1994). This alga addresses the challenge of flow-induced mechanical forces, in part, by exhibiting morphological plasticity across hydrodynamic gradients (Koehl et al. 2008). When living in hydrodynamically forceful environments, thalli develop narrow, flat blades, and when living in sheltered environments, thalli develop broad, undulate blades (Koehl and Alberte 1988, Koehl et al. 2008). The narrow-bladed morphology causes blades to compress into a streamlined cluster in flow, which reduces drag, whereas the broad-bladed morphology causes blades to flap and oscillate in flow, preventing the formation of a cluster, which increases light interception by limiting self-shading (Koehl and Alberte 1988). Development of one morphology or the other is mediated by mechanical loading imposed by drag (Gerard 1987,

Koehl et al. 2008). Due to the ability of *Nereocystis* blades to elongate at rates as high as 10-14 cm day^{-1} (Abbott and Hollenberg 1976, Kain 1987), the kelp can adjust its overall growth form extremely quickly (Koehl et al. 2008). Individual blades have their own intercalary meristems located at their bases (Nicholson 1970, Kain 1987) and the morphology of each blade can be regulated independently (Koehl et al. 2008). Overall, morphological plasticity in *Nereocystis* blades allows the kelp to maximize photosynthetic output while minimizing the amount of drag it experiences in a given environment (Koehl and Alberte 1988).

While the biomechanical consequences of kelp morphological plasticity have been well studied, there remains much about this phenomenon that is not understood. For instance, as previous experiments have primarily utilized binary "weight" and "no weight" designs (Gerard 1987, Koehl et al. 2008), we have not yet characterized a full reaction norm (how trait phenotypes vary across an environmental gradient; Woltereck 1909, Stearns 1989) of blade morphology exhibited by a *Nereocystis* across a wide range of mechanical loading. A reaction norm would provide information on how selective pressures relating to hydrodynamic forces act on blade morphology (Gibert et al. 1998, David et al. 2004). If, for example, the kelp produced a linear reaction norm of blade shape across a wide range of flow conditions, we would infer that selection on blade morphology was the same across all flow conditions and that *Nereocystis* was equally well adapted for all tested environments (Gibert et al. 1998). Alternatively, if the kelp produced a logistic reaction norm, it would suggest that selection favoured extreme phenotypes over intermediate ones and that *Nereocystis* was best suited for either very slow or very fast flow conditions (Gibert et al. 1998). Information like this may allow us to clarify the biogeographic range of *Nereocystis*, as well as enable us to predict shifts in those limits that might occur as global hydrodynamic environments continue to change (Young et al. 2011, Wang et al. 2014).

Characterizing a reaction norm would also allow us to identify limits to morphological plasticity, which could help characterize hydrodynamic constraints on the kelp. If, for instance, we saw that blade morphology stopped responding to tensile force over a certain magnitude, it might suggest that the hydrodynamic benefit of altering blade shape diminishes once a critical flow speed is exceeded.

While we know little about the range of morphologies that *Nereocystis* blades can achieve through phenotypic plasticity, we know even less about the physiological mechanisms that generate this plasticity. Previous studies by Gerard (1987) and Koehl et al. (2008) have shown that sustained tension induces kelp blades to grow narrower, longer, and less ruffled without any changes in thickness or biomass accumulation rate. Gerard (1987) hypothesized that the observed phenotypes were the result of mechanical forces inducing meristematic cells to preferentially divide in the longitudinal axis of the blade, thereby increasing elongation and reducing widening. However, this proposed mechanism has yet to be explicitly demonstrated and nothing more about the physiological processes that enable this control of cell division is known.

In contrast to kelps, land plants have a moderately well-understood set of physiological mechanisms for detecting and responding to mechanical stimuli (reviewed in Jaffe et al. 2002, Braam 2005, Telewski 2006, Chehab et al. 2008, Monshausen and Gilroy 2009, Monshausen and Haswell 2013, Sampathkumar et al. 2014, Moulia et al. 2015). It is widely believed that the initial step in plant mechanoperception is cell wall deformation (reviewed in Jaffe et al. 2002, Monshausen and Gilroy 2009). Such deformation applies tension to the plasma membrane, which initiates a signaling cascade (reviewed in Jaffe et al. 2002, Chehab et al. 2008, Monshausen and Gilroy 2009, Monshausen and Haswell 2013, Sampathkumar et al. 2014); this membrane tension and the responses it elicits can be localized and directionally specific (Gus-

Mayer et al. 1998, Louveaux et al. 2016). When the signals generated in response to mechanical stimulation ultimately influence growth and development, the entire physiological process, from stimulus detection to ultimate response, is referred to as thigmomorphogenesis (Jaffe 1973). Phytohormones are thought to be among the various signaling molecules involved in mediating this process (e.g. Erner and Jaffe 1982, Biro and Jaffe 1984, Chehab et al. 2008, 2012, Malabarba et al. 2019). These chemicals are probably the reason that thigmomorphogenetic effects can be induced in tissue regions far from where a mechanical stimulus was actually applied (Erner et al. 1980, Coutand et al. 2000). The ultimate growth responses seen in cases of plant thigmomorphogenesis are the result of changes in patterns of cell elongation and/or division (Erner et al. 1980, Louveaux et al. 2016).

Given that kelps and land plants show remarkable ecological and morphological similarity as a result of convergent evolution (Steneck et al. 2002, Keeling 2004, Drobnitch et al. 2015, Starko and Martone 2016a, Graham et al. 2017), I hypothesize that kelps and land plants might utilize common cellular features in a similarly convergent manner to address comparable evolutionary problems. If *Nereocystis* were to perceive and respond to mechanical stimuli similarly to land plants at the cellular level, I hypothesize that thalli might (1) detect such stimuli via deformation of its cell walls, (2) utilize hormones in the signal transduction cascade that followed stimulus detection, and (3) modify its blade morphology by altering meristematic cell elongation and/or division patterns. While we are not currently able to rigorously test these hypotheses due to the limited set of molecular and cell biological methods currently available for kelp systems, we can conduct experiments to better characterize the growth response of kelp blades to mechanical loading. This will help us assess which of our hypotheses, if any, are supported and might merit further examination.

In this chapter, I report on three experiments to better characterize the effect of tensile force on the growth and morphology of *Nereocystis* blades. Each involved the use of weights as a proxy for drag-induced tension (Gerard 1987, Kraemer and Chapman 1991a, Koehl et al. 2008). In the first experiment, hereafter referred to as the "load magnitude experiment", I examined how blade morphology changed across a wide gradient of tensile forces. I hypothesized that, given the broad hydrodynamic niche of *Nereocystis* (Johnson and Koehl 1994), (1) the application of a linear gradient of tensile force would yield an equally linear gradient of morphologies and (2) there would be no limits to the kelp's attainable phenotypes within the range of mechanical loading applied. In the second experiment, hereafter referred to as the "load direction experiment", I examined how the morphologies of blades and their associated meristematic cells were affected by applying tensile force to the blade transversely instead of longitudinally. I expected that the growth response yielded under high transverse loading would be rotated 90° compared to that yielded under high longitudinal loading, which would be consistent with the behaviour of cell wall-mediated thigmomorphogenetic responses observed in plants. I also predicted that effects of tension would be observed in the morphologies of both the blades and their respective cells, suggesting that tissue-level morphological changes were being driven by cell growth. In the third experiment, hereafter referred to as the "load location experiment", I investigated whether a growth response to tension could be induced in blade meristematic tissue by applying tensile force only to non-growing distal tissue. I hypothesized that applying mechanical loading to only distal tissue would not invoke plasticity in the meristematic tissue, suggesting that a long-distance signaling mechanism, such as a hormone, is unlikely to be involved.

2.3 Methods

2.3.1 Load magnitude experiment

Two mature *Nereocystis* sporophytes were collected from a surge channel located north of Brady's Beach (48°49′57″ N, 125°8′59″ W) in Bamfield, British Columbia on July 17, 2017. These kelps were brought to the Bamfield Marine Sciences Centre (BMSC) and housed in flow-through sea tables for less than 24 hours. For each of the kelps collected, five blades were haphazardly selected and cut off from the pneumatocyst such that a small piece of pneumatocyst tissue was left intact at each blade base (Fig. 2.1). All blades were cut to a standard initial length of approximately 70 cm and the morphology of each blade was characterized (Fig. 2.1). A tape measure was used to quantify midline blade length between the origin and a small hole initially punched 50 cm distal (L_B); this was measured to the nearest 1 mm. Blade width and thickness at 10 cm from the origin (W_B and T, respectively) were measured to the nearest 0.1 mm using Vernier calipers. Blade surface area (A_B) was estimated by photographing the blades and using the program ImageJ (Rasband 2019) to measure the area of the flattened projections of each blade. Ruffle (R) was quantified using the following equation:

$$R = \frac{\Sigma L_T}{\Sigma L_P} \qquad \text{Equation 2.1}$$

where L_T is the "total length" of each of the two blade edges, incorporating all ruffles and irregularities, and L_P is the flattened "projected length" of the two edges (Fig. 2.1). This method is modified from one utilized by Koehl and Alberte (1988) to permit non-destructive measurement. L_T and L_P of each blade edge were measured by laying a string along the edge from the origin to the point 50 cm from the origin as measured from the midline, then measuring the length of string laid down with a measuring tape to the nearest cm. Blade wet mass (M_B) was measured to the nearest 0.1 g after wiping excess water from tissue surfaces. The mean initial

morphological measurements across all blades (± SE) were: mean L_B = 491.9 ± 1.4 mm; mean W_B = 42.1 ± 1.5 mm; mean T = 0.6 ± 0.01 mm; mean R = 1.07 ± 0.01; mean M_B = 25.5 ± 0.9 g; mean A_B = 425.9 ± 9.6 cm^2. Mean W_B for these blades is comparable to that of kelps found in a wave- and current-sheltered environment as described by Koehl and Alberte (1988).

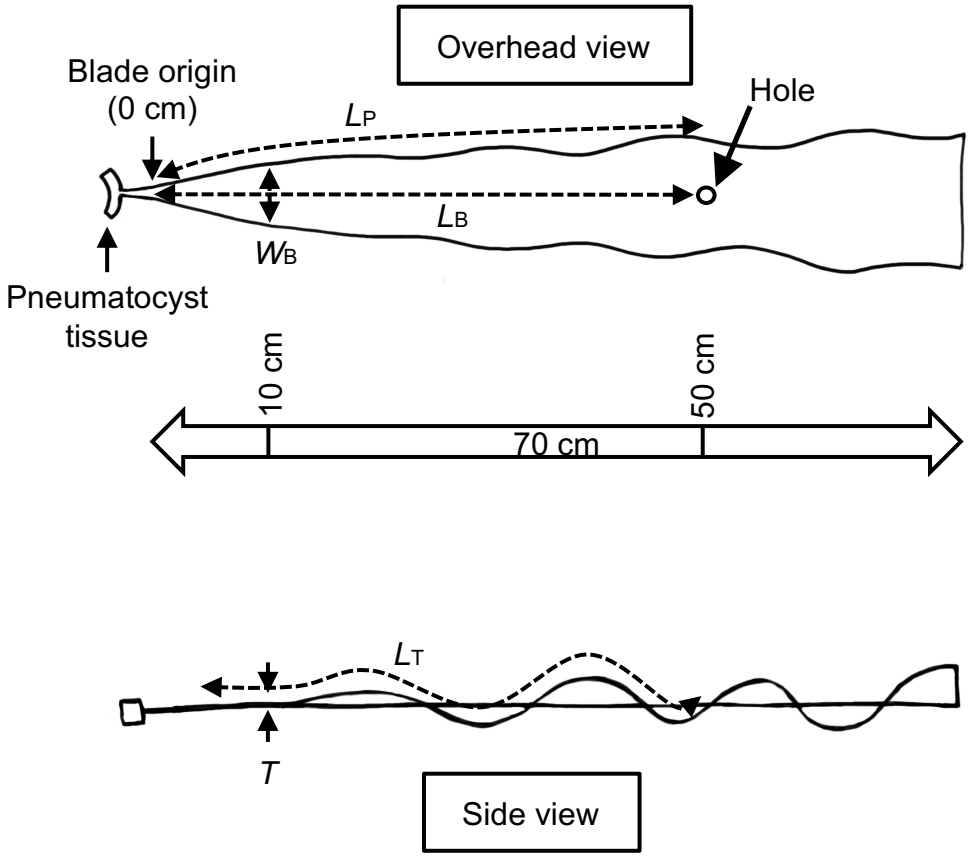

Fig. 2.1. Morphological measurements taken for experimental blades in load magnitude experiment. L_B = blade midline length; W_B = blade width at 10 cm from the origin; T = blade midline thickness at 10 cm from the origin; L_P = "projected" edge length; L_T = "total" edge length. All blades were cut to a standard initial length of 70 cm. L_B was measured between the blade origin and a small hole punched at an initial position of 50 cm distal. L_P and L_T were always measured only for the proximal 50 cm of the blade (as measured along the midline).

The most distal 15 cm of tissue of each blade was looped around a short PVC tube and the loop was sewn closed, thereby securing the tube to the blade (Fig. 2.2). This method was modified from one described in Koehl et al. (2008) for attaching weights to kelp blades. All

blades were then transferred to two outdoor growth tanks, with the blades originating from each of the two kelps being allocated to separate tanks. These tanks were positioned side by side and exposed to direct ambient sunlight. Fresh seawater was continuously pumped into the tanks from the bottom of the nearby Grappler Inlet. This water was consistently between 10 and 12°C and had a salinity of 35 ppt. Fast incoming flow was not pointed directly at the experimental blades in order to minimize additional mechanical loading being applied to growing tissues, but blades were still exposed to low levels of water motion.

To secure kelp blades into the growth tanks, the proximal ends of each blade were attached to PVC bars suspended below water by wrapping cable ties around the intact pieces of pneumatocyst. The tubes attached to the distal ends of each blade were then connected to free-hanging weights by monofilament lines that extended horizontally across the tanks, then, aided by a set of pulleys, up and over the tank edges (Fig. 2.2). Each of the six blades in the two tanks had a different amount of weight attached (0, 0.5, 1.0, 1.5, 2.0, and 2.5 N). These weight levels were chosen to represent a range of loading that *Nereocystis* blades might naturally experience in flow due to drag as assessed by our own measurements of drag on single *Nereocystis* blades (Coleman unpublished data) and observations that local populations can experience current velocities in excess of 3 m s^{-1} (Canadian Hydrographic Service 2017). The position of each weight treatment within each tank was random. The blades were left in place to grow under constant longitudinal tension imposed by the weights for 4-5 days.

At the end of the growth period, the experimental blades were removed from the growth tanks and all morphological parameters were re-measured. All methods up to this point were then repeated for two more *Nereocystis*. Once the final measurements were collected for the additional kelps, ordinary least squares (OLS) regression was used to assess the effect of weight

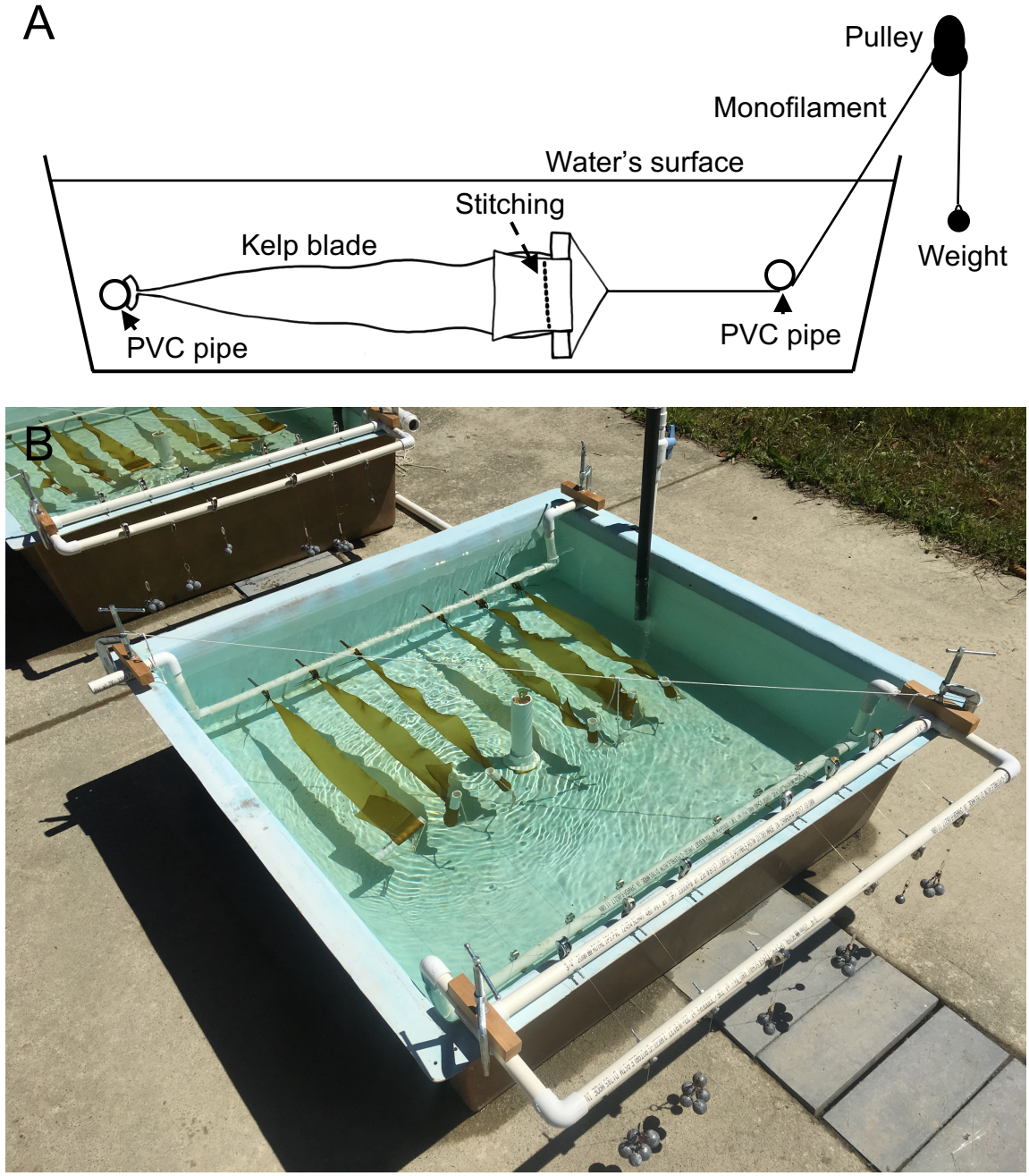

Fig. 2.2. (A) Diagram and (B) photograph of experimental setup used in load magnitude experiment.

on all measured variables (expressed as percent change day^{-1}; ΔL_B, ΔW_B, ΔT, ΔR, ΔM_B, and ΔA_B). Kelp of origin was incorporated into regression analyses as an interactive fixed effect due

to the low number of individual kelps used. Additionally, the stress (in MN m^{-2}) experienced by each blade at the beginning and end of its growth period was calculated using the following equation:

$$Stress = \frac{Weight}{0.25\pi W_B T} \qquad \text{Equation 2.2}$$

Blade cross-sectional area was modeled as an ellipse since the margins of kelp blades are known to be thinner than the midlines (Gerard 1987). To see if stress changed over the course of experimental growth periods as blade morphology changed, a linear model was used to test whether change in stress (in % day^{-1}) across all weight treatments except the control group was significantly different from zero. OLS regression was also used to test whether the amount of weight applied (above 0 N) had a significant effect on change in stress. Finally, in order to check whether load was a useful predictor of kelp response, OLS regressions were used to assess the effect of stress (averaged between the beginning and end of growth periods) on ΔL_B, ΔW_B, ΔT, ΔR, ΔM_B, and ΔA_B; kelp of origin was incorporated into these regression models as a fixed interactive covariate.

All statistical analyses were performed in R (R Core Team 2021). All OLS regressions were performed using the lm() function from the R stats package (R Core Team 2021). ANOVA was performed on regression models using the anova() function from the R stats package (R Core Team 2021). Assumptions of linearity were verified with residuals vs. fitted plots generated using the plot() function from the R graphics package (R Core Team 2021). Assumptions of normality were verified with Shapiro-Wilk tests performed with the ols_test_normality() function from the olsrr package (Hebbali 2020). Assumptions of homoscedasticity were verified with Breusch-Pagan tests performed with the ols_test_breusch_pagan() function from the olsrr package (Hebbali 2020). Assumptions of independence of residuals were verified with Durbin-

Watson tests performed with the durbinWatsonTest() function from the car package (Fox and Weisberg 2019). No data were found to deviate from any underlying assumptions of the analyses performed.

2.3.2 Load direction experiment

A single blade was haphazardly selected and removed at its base from each of 40 mature *Nereocystis* sporophytes growing at Stanley Park (49°18′10″ N, 123°07′35″ W) and brought to the University of British Columbia (UBC) on June 27, 2018. Collected blades were stored in a sea table for up to 48 hours. An approximately 10 x 10 cm square of tissue was cut out of each blade at the most proximal position possible; a small notch was cut into the centre of the distal edge of each of these tissue samples to denote the orientation of the original blade. Tissue samples were weighed to the nearest 0.1 g and photographed. The software ImageJ (Rasband 2019) was used to measure the midline length (L_S) and width (W_S) to the nearest 0.1 cm, as well as the surface area (A_S) to the nearest 0.1 cm^2, of each tissue sample (Fig. 2.3).

For each kelp tissue sample, two opposite edges were looped around short plastic rods and the loops were sewn closed. For 20 of the 40 samples, hereafter referred to as the "longitudinal" treatment group, the proximal and distal edges were folded over, while for the other 20, hereafter referred to as the "transverse" treatment group, the left and right edges were folded (Fig. 2.3). All tissue samples had weights attached to one of the two plastic rods; in the longitudinal group, the weight was always attached to the proximal rod. 10 of the 20 samples from each treatment group had a 1.8 N weight attached (the "high weight" group), while the remaining samples had 0.28 N weights attached (the "low weight" group). Each tissue sample's non-weight-bearing rod was tied to a PVC tube positioned over a growth tank such that the

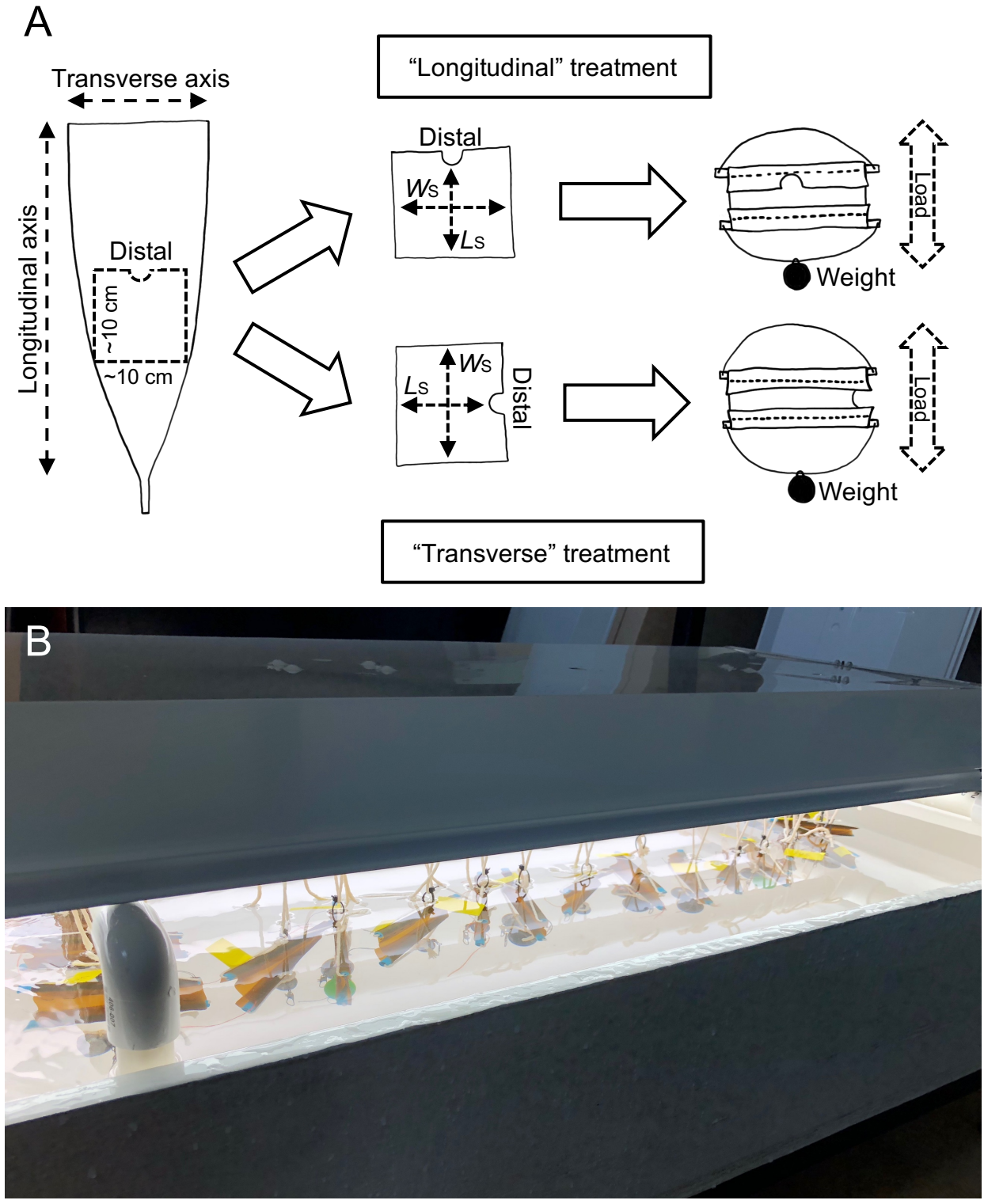

Fig. 2.3. (A) Methods for preparation of kelp tissue samples in load direction experiment. L_S = tissue sample length. W_S = tissue sample width. (B) Photograph of experimental setup illustrated in (A).

samples were suspended in mid water with their respective weights applying constant tension in either the longitudinal or transverse axis with respect to the original blades (Fig. 2.3). Tissues were left to grow for 6 days. The growth tank was maintained at a temperature of 10-11°C and a salinity of 31 ppt; kelps received 300-400 μmol m^{-2} s^{-1} of photons over an 18:6 photoperiod and were cultured in half-strength f/2 growth medium. Water was continuously pumped through the tanks to maintain a low level of ambient motion; fast incoming flow was never pointed directly at experimental samples.

At the end of the growth period, the kelp tissue samples were re-weighed and re-photographed; final L_S, Ws, and A_S were measured in ImageJ (Rasband 2021). An approximately 1x1 cm^2 subsample was taken from the centre of each tissue sample and fixed in 5% formalin seawater for histological examination. An Olympus BX51W1 microscope was used to image the top layer of meristoderm cells in each subsample. Photographs were taken of three random locations within each subsample using an Olympus DP21 camera. ImageJ was used to measure the area, length, and width of all meristoderm cells in each photograph. The means of these three variables were calculated for each photograph and these mean values were averaged across the three subsamples to yield measures of mean meristoderm cell length (L_C), width (W_C), and area (A_C) for each kelp square. Cell length and width were measured using the bounding rectangle method in order to maintain a fixed orientation with respect to that of the original blades (Fig. 2.3).

All data analysis was performed in R (R Core Team 2021). Two-factor ANOVA was used to analyze the effects of weight and orientation, as well as an interaction between the two, on kelp tissue and cell morphology data. Tissue morphology variables (wet mass (M_S), L_S, A_S, and W_S) were expressed as percent change day^{-1} (ΔM_S, ΔL_S, ΔA_S, and ΔW_S respectively). PCA

was used to identify axes of maximal variation in ΔM_S, ΔL_S, ΔA_S, and ΔW_S; two-factor ANOVA was used to test the effect of weight and orientation on resulting principal component scores. All ANOVAs were performed using the lm() and anova() functions from the R stats package (R Core Team 2021). Assumptions of normality were verified with Shapiro-Wilk tests performed with the ols_test_normality() function from the olsrr package (Hebbali 2020). Assumptions of homoscedasticity were verified using F-tests peformed with the ols_test_f() function from the olsrr package (Hebbali 2020). Assumptions of independence of residuals were verified with Durbin-Watson tests performed with the durbinWatsonTest() function from the car package (Fox and Weisberg 2019). Data were not found to deviate from any underlying assumptions of the analyses performed. Tukey-Kramer post-hoc tests were performed using the lsmeans() function from the emmeans package (Lenth 2020) to identify significantly different treatment groups when any ANOVA identified significant interaction effects. PCA was performed with the prcomp() function from the R stats package (R Core Team 2021); data were centered and scaled.

Standard major axis (SMA) regression was used to assess whether L_C and W_C were significant predictors of final L_S and W_S respectively; this was performed using the lmodel2() function from the lmodel2 package (Legendre 2018). Assumptions of normality were verified with Shapiro-Wilk tests performed with the shapiro.test() function from the R stats package (R Core Team 2021). Assumptions of homoscedasticity were verified using Breusch-Pagan tests performed using the bptest() function from the lmtest package (Zeileis and Hothorn 2002). Assumptions of independence of residuals were verified with Durbin-Watson tests performed with the dwtest() function from the lmtest package (Zeileis and Hothorn 2002). Data were not found to deviate from any underlying assumptions of the analyses performed.

2.3.3 Load location experiment

One *Nereocystis* blade was haphazardly selected and removed at the base from each of 20 mature kelps growing near Brockton Point lighthouse in Stanley Park (49°17′56″ N, 123°17′56″ W) and brought to UBC on May 8, 2019. The collected blades were stored in a sea table for up to 72 hours. Each blade was cut to a standard initial midline length of approximately 50 cm and W_B was measured to the nearest 0.1 mm using Vernier calipers. Mean initial W_B across all blades (± SE) was 54.7 ± 2.1 mm, which is higher than that of kelps found in a wave- and current-sheltered environment as described by Koehl and Alberte (1988). Both the proximal and distal ends of each blade were looped around short plastic rods; these loops were sewn closed (Fig. 2.4). At the distal end, approximately 2 cm of tissue was folded over, while at the proximal end, 15 cm of tissue was.

Experimental blades were secured into two growth tanks. The proximal ends of the blades were tied to a PVC pipe via the plastic rod sewn into the tissue; this was done such that the most proximal 15 cm of blade tissue was always left slack. The distal end of each blade was tied, via the other plastic rod, to a line of monofilament that extended horizontally; these lines ultimately connected the experimental blades to weights hanging off the ends of the growth tanks, as in Fig. 2.2. The weights applied differing degrees of loading to only the most distal ~35 cm of each blade; 10 of the 20 blades experienced 0.17 N of constant longitudinal tension (the "low weight" group), while the other 10 received 1.0 N (the "high weight" group). Five blades from each weight treatment group were assigned to each of the two growth tanks; blades in each tank were positioned randomly. Kelp tissue was left in place to grow for 5 days. Water was continuously pumped through the tanks to maintain a low level of ambient motion; fast incoming flow was never pointed directly at experimental samples.

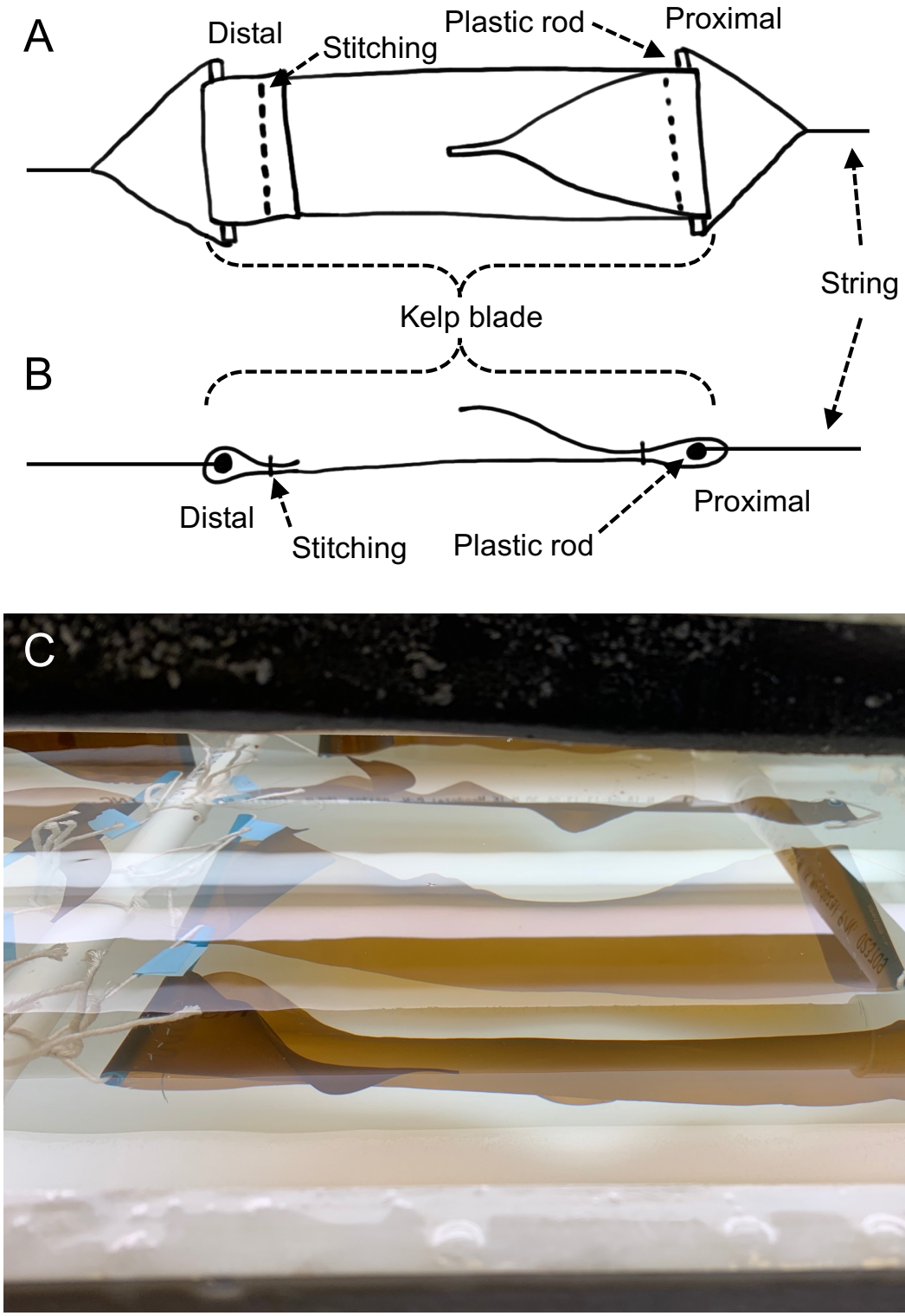

Fig. 2.4. Experimental methods for load location experiment. (A) Kelp blade configuration as viewed from above; (B) kelp blade configuration as viewed from the side; (C) photograph of experimental setup illustrated in (A) and (B).

At the end of the growth period, W_B was re-measured. The effect of weight on ΔW_B was assessed statistically using a two-way ANOVA; growth tank was incorporated into the analysis as an interactive fixed effect due to the low number of tanks. All data analysis was performed in R (R Core Team 2021). ANOVA was performed using the lm() and anova() functions from the R stats package (R Core Team 2021). Assumptions of normality were verified with Shapiro-Wilk tests performed with the ols_test_normality() function from the olsrr package (Hebbali 2020). Assumptions of homoscedasticity were verified using F-tests peformed with the ols_test_f() function from the olsrr package (Hebbali 2020). Assumptions of independence of residuals were verified with Durbin-Watson tests performed with the durbinWatsonTest() function from the car package (Fox and Weisberg 2019). Data were not found to deviate from any underlying assumptions of the analyses performed.

2.4 Results

2.4.1 Load magnitude experiment

Blades bearing higher amounts of weight grew significantly longer (ΔL_B; ANOVA, $F_{1,13}$=16.1, p=0.001) and heavier (ΔM_B; ANOVA, $F_{1,13}$=4.98, p=0.044), as well as significantly narrower (ΔW_B; ANOVA, $F_{1,13}$=23.3, p<0.001) and less ruffled (ΔR; ANOVA, $F_{1,13}$=11.4, p=0.005), compared to those bearing lower amounts of weight (Table 2.1; Fig. 2.5). These changes in morphology appeared continuous and approximately linear across the loading gradient. There were no significant effects of weight on blade thickness (ΔT; ANOVA, $F_{1,13}$=0.66, p=0.43) or blade area (ΔA_B; ANOVA, $F_{1,13}$=4.27, p=0.06). There were no significant effects of kelp individual of origin or weight:kelp interactions on any measured variables (Table

Table 2.1. ANOVA tables for load magnitude experiment

Response	Explanatory	df	Sum Sq	Mean Sq	F	p
Change in blade length (ΔL_B)	Weight	1	54.3	54.3	16.1	0.001
	Kelp	3	12.4	4.14	1.22	0.34
	Weight:Kelp	3	8.87	2.96	0.88	0.48
	Residuals	13	43.8	3.37		
Change in blade width (ΔW_B)	Weight	1	102.3	102.3	23.3	<0.001
	Kelp	3	30.5	10.2	2.32	0.13
	Weight:Kelp	3	26.3	8.75	2.00	0.16
	Residuals	13	57.0	4.38		
Change in blade thickness (ΔT)	Weight	1	3.20	3.20	0.66	0.43
	Kelp	3	6.23	2.08	0.43	0.73
	Weight:Kelp	3	6.87	2.29	0.48	0.70
	Residuals	13	62.6	4.82		
Change in ruffle (ΔR)	Weight	1	2.79	2.79	11.4	0.005
	Kelp	3	2.22	0.74	3.04	0.067
	Weight:Kelp	3	0.17	0.056	0.23	0.87
	Residuals	13	3.17	0.24		
Change in blade wet mass (ΔM_B)	Weight	1	12.0	12.0	4.98	0.044
	Kelp	3	15.9	5.31	2.20	0.14
	Weight:Kelp	3	4.47	1.49	0.62	0.62
	Residuals	13	31.4	2.42		

Change in blade area (ΔA_B)	Weight	1	16.8	16.8	4.27	0.059
	Kelp	3	24.1	8.02	2.04	0.16
	Weight:Kelp	3	27.2	9.05	2.31	0.12
	Residuals	13	51.0	3.92		

2.1). All but one sample of the 2.5 N treatment broke in the first 24 hours of their growth periods; no sample loss occurred at other weight levels. In all instances where blade tissue failed, the failure occurred in the narrow region near the blade base, distal to the pneumatocyst; breaks never took place near the stitching securing the blades to the weights.

The amount of stress (N m^{-2}) experienced by the kelp blades was found to not be constant throughout the experiment (linear model, $p<0.001$), but the amount that stress changed was not found to differ between weight treatments (ANOVA, $F_{1,9}=2.81$, $p=0.13$) or kelps of origin (ANOVA, $F_{3,9}=1.91$, $p=0.20$; see supplemental Table A1, Fig. A1). There were no significant effects of weight:kelp interactions on change in stress (ANOVA, $F_{3,9}=0.54$, $p=0.66$). Effects of mean stress on kelp morphological variables were found to be, for the most part, identical to the effects of weight (see supplemental Table A2, Fig. A2). The only exceptions were an instance where mean stress was found to cause a significant change in blade area (ΔA_B; ANOVA, $F_{1,13}=5.55$, $p=0.035$) when weight did not and another instance where the ruffle (ΔR) was found to change to a significantly different degree between kelps of origin (ANOVA, $F_{3,13}=3.83$, $p=0.036$) only when mean stress was incorporated into the regression model instead of weight.

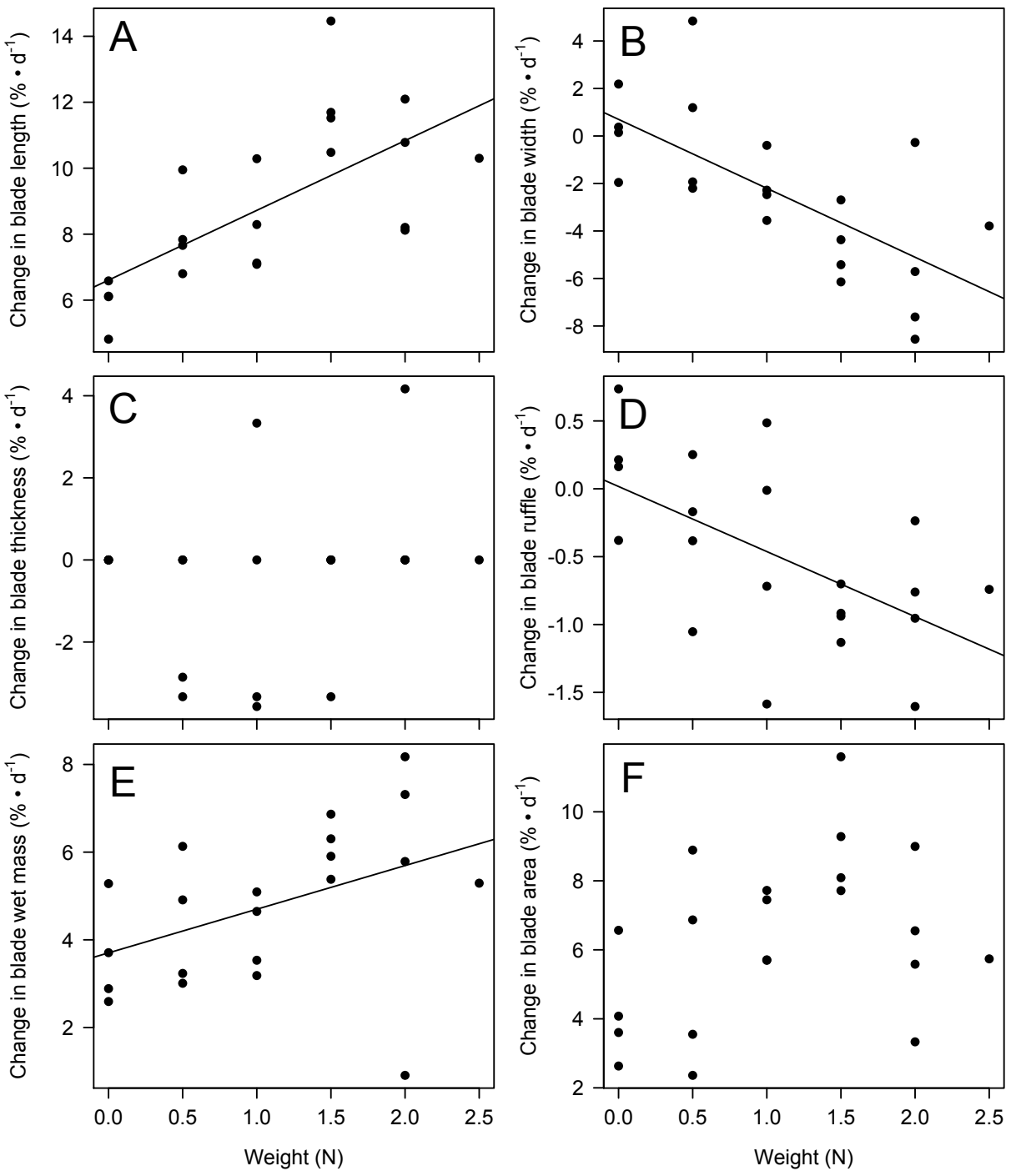

Fig. 2.5. Change in morphological characters of *Nereocystis* blades expressed as functions of weight applied during load magnitude experiment. OLS regression lines indicate a statistically significant relationship between weight and the specified response variable. Change in blade length = ΔL_B; change in blade width = ΔW_B; change in blade midline thickness = ΔT; change in blade ruffle = ΔR; change in blade wet mass = ΔM; change in blade area = ΔA.

2.4.2 Load direction experiment

There was no significant effect of weight on change in kelp tissue length (ΔL_S; ANOVA, $F_{1,33}$=0.68, p=0.41), width (ΔW_S; ANOVA, $F_{1,33}$=1.18, p=0.29), area (ΔA_S; ANOVA, $F_{1,33}$=0.85, p=0.36), or wet mass (ΔM_S; ANOVA, $F_{1,33}$=0.011, p=0.92; Table 2.2; see supplemental Fig. A3). There was no significant effect of kelp tissue orientation on change in tissue sample length (ΔL_S; ANOVA, $F_{1,33}$=0.049, p=0.83), width (ΔW_S; ANOVA, $F_{1,33}$=1.34, p=0.26), area (ΔA_S; ANOVA, $F_{1,33}$=0.64, p=0.43), or wet mass (ΔM_S; ANOVA, $F_{1,33}$=1.60, p=0.21). There was a significant effect of an interaction between weight and orientation on change in tissue sample length (ΔL_S; ANOVA, $F_{1,33}$=7.85, p=0.008), but not on change in tissue sample width (ΔW_S; ANOVA, $F_{1,33}$=0.077, p=0.78), area (ΔA_S; ANOVA, $F_{1,33}$=1.21, p=0.28), or wet mass (ΔM_S; ANOVA, $F_{1,33}$=3.39, p=0.074). No pairwise combination of weight and orientation treatment groups were found to significantly differ in elongation rate (ΔL_S), but the high weight, transversely oriented kelp tissue samples elongated nearly significantly less than the low weight, transversely oriented samples (Tukey, p=0.061).

The first principal component (PC1) was positively correlated with all four input variables and was interpreted as an index of overall growth. The second principal component (PC2) was negatively correlated with elongation (ΔL_S), but positively correlated with widening (ΔW_S); it was interpreted as an index of shape change. PC1 explained 91.4% of the variation in ΔL_S, ΔW_S, ΔA_S, and ΔM_S, while PC2 explained 5.6% (Table 2.3).

There was no significant effect of weight (ANOVA, $F_{1,33}$=0.57, p=0.45), orientation (ANOVA, $F_{1,33}$=0.80, p=0.38), or an interaction between the two (ANOVA, $F_{1,33}$=1.89, p=0.18) on overall growth (PC1 scores; Table 2.4; Fig. 2.6). There was no significant effect of weight

Table 2.2. ANOVA tables for tissue morphology data from load direction study

Response	Explanatory	df	Sum Sq	Mean Sq	F	p
Change in tissue sample length (ΔL_S)	Weight	1	1.21	1.21	0.68	0.41
	Orientation	1	0.086	0.086	0.049	0.83
	Weight:Orientation	1	13.9	13.9	7.85	0.008
	Residuals	33	58.5	1.77		
Change in tissue sample width (ΔW_S)	Weight	1	1.91	1.91	1.18	0.29
	Orientation	1	2.18	2.18	1.34	0.26
	Weight:Orientation	1	0.13	0.12	0.077	0.78
	Residuals	33	53.6	1.63		
Change in tissue sample area (ΔA_S)	Weight	1	7.01	7.01	0.85	0.36
	Orientation	1	5.26	5.26	0.64	0.43
	Weight:Orientation	1	10.0	10.0	1.21	0.28
	Residuals	33	273.0	8.27		
Change in tissue sample wet mass (ΔM_S)	Weight	1	0.081	0.081	0.011	0.92
	Orientation	1	12.0	12.0	1.60	0.21
	Weight:Orientation	1	25.3	25.3	3.39	0.074
	Residuals	33	246.4	7.47		

(ANOVA, $F_{1,33}$=1.79, p=0.19) or orientation (ANOVA, $F_{1,33}$=3.05, p=0.090) on shape change (PC2 scores), but there was a significant interaction effect (ANOVA, $F_{1,33}$=46.5, p<0.001) on shape change. The low weight, longitudinally oriented tissue samples grew significantly longer and narrower than the high weight, longitudinally oriented tissue samples (Tukey, p<0.001),

Table 2.3. Output of PCA performed for load direction experiment

Variable	Loadings			
	PC1	PC2	PC3	PC4
Change in square length (ΔL_S)	0.493	-0.619	-0.461	-0.402
Change in square width (ΔW_S)	0.489	0.741	-0.076	-0.454
Change in square area (ΔA_S)	0.518	0.115	-0.292	0.795
Change in square wet mass (ΔM_S)	0.499	-0.235	0.834	0.016
Proportion of variation	0.913	0.056	0.028	0.002

Table 2.4. ANOVA tables for principal component scores generated from tissue morphology data in load direction study

Response	Explanatory	df	Sum Sq	Mean Sq	F	p
Principal component 1 (PC1; growth)	Weight	1	2.08	2.08	0.57	0.45
	Orientation	1	2.89	2.89	0.80	0.38
	Weight:Orientation	1	6.85	6.85	1.89	0.18
	Residuals	33	119.7	3.63		
Principal component 2 (PC2; -elongation, widening)	Weight	1	0.17	0.17	1.79	0.19
	Orientation	1	0.29	0.29	3.05	0.09
	Weight:Orientation	1	4.46	4.46	46.5	<0.001
	Residuals	33	3.16	0.10		

whereas among the transversely oriented tissue samples, the low weight individuals grew significantly shorter and wider than the high weight ones (Tukey, $p<0.001$).

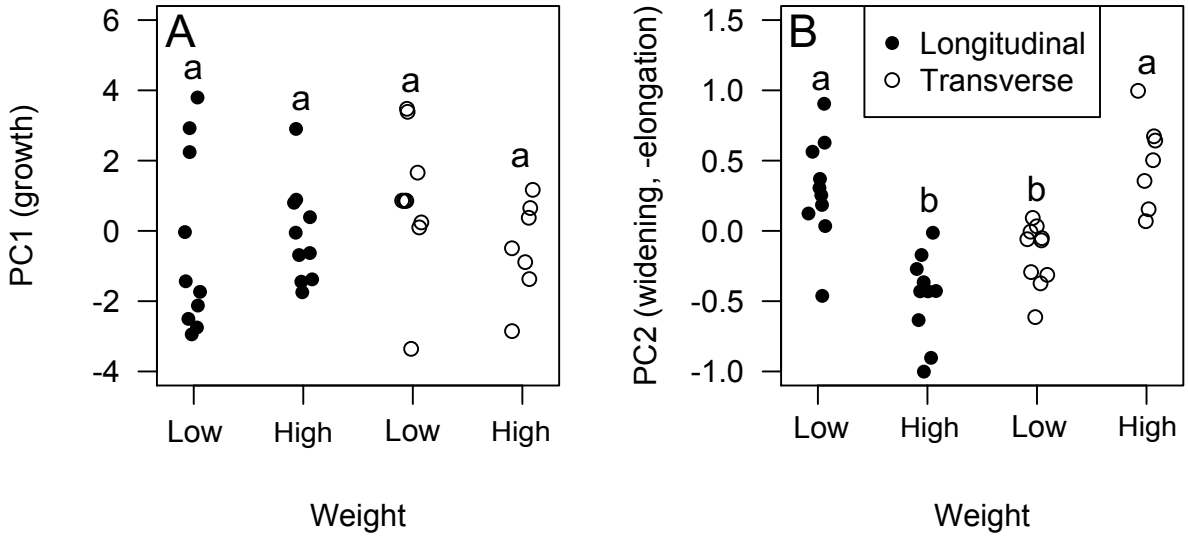

Fig. 2.6. Principal component 1 (PC1) and principal component 2 (PC2) scores generated from PCA performed for stress direction experiment expressed as functions of weight level applied and orientation of kelp squares; lowercase letters denote significantly different groups as indicated by a Tukey–Kramer post hoc test.

There was no significant effect of weight on mean meristoderm cell length (L_C; ANOVA, $F_{1,33}$=2.46, p=0.13), width (W_C; ANOVA, $F_{1,33}$=0.11, p=0.74), or area (A_C; ANOVA, $F_{1,33}$=3.45, p=0.072; Table 2.5; see supplemental Fig. A4). There was no significant effect of orientation on mean meristoderm cell length (L_C; ANOVA, $F_{1,33}$=2.43, p=0.13) or area (A_C; ANOVA, $F_{1,33}$=0.61, p=0.44), but transversely oriented tissue samples had significantly wider meristoderm cells than longitudinally oriented ones across both weight levels (ANOVA, $F_{1,33}$=4.84, p=0.035). There was a significant effect of an interaction between weight and orientation on mean meristoderm cell length (L_C; ANOVA, $F_{1,33}$=8.67, p=0.006), but not on mean meristoderm cell width (W_C; ANOVA, $F_{1,33}$=0.015, p=0.90) or area (A_C; ANOVA, $F_{1,33}$=1.53, p=0.23). The heavily weighted, longitudinally oriented tissue samples had significantly longer meristoderm cells than both the lightly weighted, longitudinally oriented tissue samples (Tukey, p=0.023) and

Table 2.5. ANOVA tables for cell morphology data from load direction experiment

Response	Explanatory	df	Sum Sq	Mean Sq	F	p
Mean cell length (L_C)	Weight	1	0.31	0.31	2.46	0.13
	Orientation	1	0.31	0.31	2.43	0.13
	Weight:Orientation	1	1.11	1.11	8.67	0.006
	Residuals	33	4.22	0.13		
Mean cell width (W_C)	Weight	1	0.012	0.012	0.11	0.74
	Orientation	1	0.54	0.54	4.84	0.035
	Weight:Orientation	1	0.002	0.002	0.015	0.90
	Residuals	33	3.65	0.11		
Mean cell area (A_C)	Weight	1	25.5	25.5	3.45	0.072
	Orientation	1	4.49	4.49	0.61	0.44
	Weight:Orientation	1	11.3	11.3	1.53	0.23
	Residuals	33	243.9	7.39		

the heavily weighted, transversely oriented tissue samples (Tukey, p=0.014). See supplemental Fig. A5 for distributions of cell morphologies in different treatment groups.

Final kelp tissue sample length (L_S) was found to significantly increase with mean meristoderm cell length (L_C; SMA regression, $p<0.001$). Final kelp tissue sample width (W_S) was found to significantly increase with mean meristoderm cell width (W_C; SMA regression, p=0.012; Fig. 2.7).

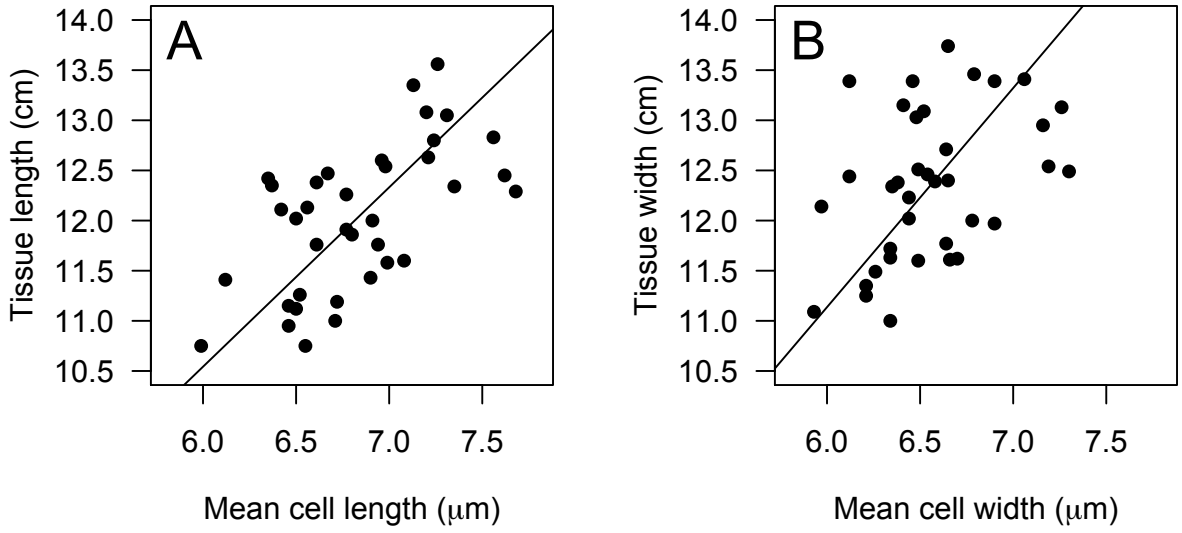

Fig. 2.7. Final tissue sample (A) length (L_S) and (B) width (W_S) expressed as functions of final mean meristoderm cell length and width respectively for kelp tissue samples in load direction experiment. Standard major axis (SMA) regression lines indicate that cell lengths and widths are statistically significant predictors of tissue lengths and widths respectively.

2.4.3 Load location experiment

There was no significant effect of weight (ANOVA, $F_{1,13}$=0.41, p=0.54), growth tank (ANOVA, $F_{1,13}$=2.10, p=0.17), or weight:growth tank interactions (ANOVA, $F_{1,13}$=1.24, p=0.29) on change in blade width (ΔW_B; Table 2.6; Fig. 2.8).

Table 2.6. ANOVA table for load location experiment

Response	Explanatory	df	Sum Sq	Mean Sq	F	p
Change in blade width (ΔW_B)	Weight	1	1.25	1.25	0.41	0.54
	Tank	1	6.47	6.47	2.11	0.17
	Weight:Tank	1	3.81	3.81	1.24	0.29
	Residuals	13	40.0	3.08		

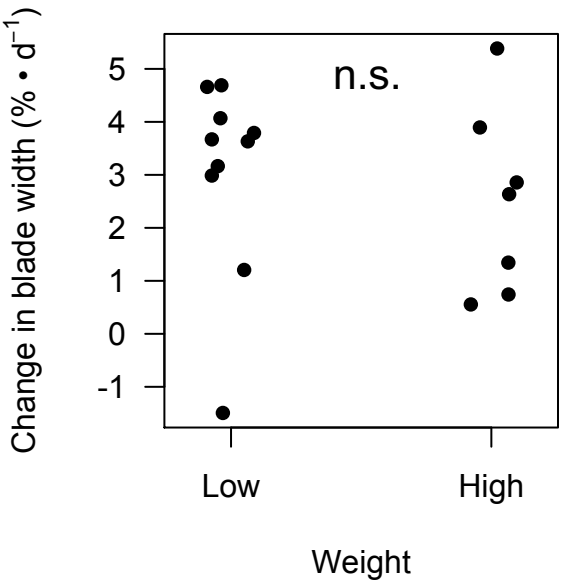

Fig. 2.8. Change in blade width (ΔL_B) expressed as a function of weight level applied during growth period in load location experiment; n.s. = not significantly different.

2.5 Discussion

Morphological plasticity across hydrodynamic gradients in the blades of *Nereocystis* has been recognized for some time and its functional significance has been well studied (Koehl and Alberte 1988, Johnson and Koehl 1994). However, the nature of the growth response to tensile force that facilitates this phenomenon, including underlying physiological mechanisms, remains poorly understood. To better characterize these, I examined how morphological plasticity in *Nereocystis* blades was affected by the (1) magnitude, (2) direction, and (3) location of mechanical loading, using weights to simulate drag imposed on the blades by moving water.

2.5.1 *Nereocystis* is sensitive to the magnitude of mechanical loading

As more tensile force was applied, experimental blades became longer, narrower, less ruffled, and heavier, but did not change in thickness and increased similarly in area across loading treatments. Furthermore, weight-induced changes in morphology scaled continuously and, especially in the case of blade width, approximately linearly with increasing loading. In other words, the reaction norms of measured blade characteristics across a loading gradient were linear, consistent with our hypothesis. Thus, *Nereocystis* is able to perceive even minor changes in the ambient mechanical environment and respond proportionally, which presumably allows it to precisely optimize its hydrodynamic and photosynthetic performance for a given flow environment (see Koehl and Alberte 1988). Such phenotypic flexibility, coupled with exceptionally high growth rates (Abbott and Hollenberg 1976, Kain 1987), may be a key factor in the observed ability of *Nereocystis* to successfully colonize a wide range of hydrodynamic environments (Koehl and Alberte 1988) on an annual basis (Abbott and Hollenberg 1976). My results are consistent with predictions from the land plant literature which suggest that plasticity is a favourable adaptive strategy in annual plants living in variable environments (Cook and Johnson 1968, Wilken 1977, Zangerl and Bazzaz 1983), especially when organismal response times to environmental changes are short (Padilla and Adolph 1996, Alpert and Simms 2002). The linear reaction norms observed suggest that selective pressure on *Nereocystis* blade morphology is equal across the loading gradient tested and that this kelp has no specific set of flow conditions within its hydrodynamic niche for which it is "best" adapted (Gibert et al. 1998, David et al. 2004).

Even though I found that stress was not constant throughout the growth periods due to progressive narrowing of blades, the facts that (1) individual weight treatments did not

significantly differ in the magnitude of stress change observed and (2) the overall results of this experiment were almost identical depending on whether weight or stress were used in my analyses suggest that mechanical load is a useful predictor of kelp plasticity in this instance.

Because blades in the 2.5 N treatment group were close to their known mechanical limits (Johnson and Koehl 1994, Hale 2001) and almost all of these individuals broke within 24 hours of their growth periods, I conclude that the range of weights used in this experiment covered the full breadth of tensile force that the blades could have withstood. The observation that blade morphology continued to respond to increasing levels of tension all the way up to the mechanical limits of the tissue suggests that there is no practical limit to the plasticity and that morphological adjustments are still functionally relevant even at high levels of water motion. Furthermore, given the linear nature of the reaction norms, I expect that subjecting blades to loading levels beyond those tested here would cause them to narrow even further if material properties had permitted. This interplay between hydrodynamic performance and material properties of blades is interesting and deserves further study, as recent work has demonstrated a clear trade-off between drag avoidance and drag tolerance in kelps (Starko and Martone 2016b). Drag avoidance via blade narrowing may compensate for the relative weakness of kelp tissues (Hale 2001, Martone 2007), but ultimately may be constrained by these material properties. On the other hand, the benefits of blade narrowing may be limited at fast water velocities (Milligan and DeWreede 2004, de Bettignies et al. 2013), so strengthening tissues to permit further narrowing would likewise not be beneficial.

As mechanical loading was applied constantly and unidirectionally in this experiment, my results are best considered to be reflective of how *Nereocystis* would behave when subjected primarily to strong currents. Wave-swept kelps, in comparison, would likely experience greater

loading forces, but these forces would be applied transiently and repeatedly (Koehl 1984, Denny et al. 1998, Gaylord et al. 2008). It would be interesting to consider how my results might differ if we were to incorporate a punctuated loading regime such as this into my experimental design. Johnson and Koehl (1994) have shown that wave-exposed *Nereocystis* show a mean blade morphology that is intermediate to those of current-swept and sheltered individuals. This may indicate that wave-imposed mechanical loading does affect the kelp's blade morphology, but to a lesser degree than current-imposed loading. This could suggest that blade morphology might be best predicted by the average mechanical loading experienced over a given time period. Based on this, an experiment incorporating transient, repeated mechanical loading treatments might be expected to produce results similar to those described here, but with shallower reaction norm slopes.

An unexpected finding of this experiment was that more highly weighted blades showed greater increases in wet mass than less weighted ones in spite of there being no significant effects of weight on blade area or thickness (and therefore volume). I see several possible explanations for this. One is that highly weighted kelps produced heavier tissue than the less weighted individuals. This could have been brought about by high loading inducing blades to incorporate more carbon into their cell walls, as described by Kraemer and Chapman (1991). Another possibility is that the morphological changes brought about by high tensile force facilitated an increase in productivity and growth (Gerard and Mann 1979, Koehl and Alberte 1988), resulting in an indirect positive effect of mechanical loading on wet mass accumulation rate. It is also possible that the additional wet mass found in highly weighted kelps could have been accounted for by thickened blade margins, which would have gone undetected in our measurements of blade midline thickness. However, I consider this unlikely, as Gerard (1987) found that

mechanical loading had no effect on blade thickness as measured at either the midlines or the margins in *Saccharina latissima*. While I cannot explain my observations with certainty, I strongly feel that they merit further study. It would be of particular significance if mechanical loading directly increases kelp tissue mass via a mechanism similar to that proposed by Kraemer and Chapman (1991), as researchers have long been interested in relationships between water motion and primary productivity (reviewed in Hurd 2000).

My biomass observations contrast with those of Gerard (1987), who found that *Saccharina latissima* blades became longer and narrower, but did not accumulate more biomass, when grown subject to high tensile force. This discrepancy may be due to methodological differences between the two studies. My data include measurements of total wet mass accumulated after 4-5 days, whereas Gerard's data are estimates of biomass production calculated based on weight:length ratios measured during the sixth week of her experiment. Based on this, it is possible, for example, that I detected a proportional increase in biomass that only occurs for a brief period after the loading is first applied, or that Gerard's calculations systematically underestimated the biomass actually accumulated by highly weighted kelps.

2.5.2 *Nereocystis* is sensitive to the direction of mechanical loading

My data indicate that the application of tension to *Nereocystis* blade tissue encourages growth in whichever axis is parallel to the tensile force while discouraging growth in the perpendicular axis. This is consistent with my hypothesis and has several important implications for my thinking on mechanisms facilitating morphological plasticity in *Nereocystis*. Firstly, it suggests that there is no substantial separation between the location of stimulus perception and that of the ultimate organismal response, which, coupled with the known ability of cell wall-

mediated thigmomorphogenetic responses in plants to be directionally sensitive (Gus-Mayer et al. 1998, Sampathkumar et al. 2014a, Louveaux et al. 2016), leads me to hypothesize that the entire set of physiological processes that facilitate morphological plasticity in *Nereocystis* blades takes place within individual cells and that cell wall deformation is the likely basis for the mechanoperception mechanism. Secondly, the lack of separation between the locations of stimuli and responses suggests that hormones (or some other long-distance signaling molecule) are unlikely to be involved in this process. I suspect that mechanical loading on the blade distends the walls of meristematic cells, initiating a signaling cascade that probably only serves to influence growth and/or division of those same cells.

While I cannot state definitively from the data at hand exactly how cell growth and division are affected by tension in this system, it does appear that growth of the tissue is somehow reallocated from the axis perpendicular to that of the tensile force into the axis parallel with it. This is consistent with, but does not explicitly confirm, the conclusions of Gerard (1987), who proposed that longitudinal tension caused meristematic cells of *Saccharina latissima* to preferentially divide in the longitudinal axis over the transverse axis. Additionally, the final morphology of the experimental kelp tissues is reflective of the average morphology of those tissues' meristoderm cells, with longer tissues, for instance, also exhibiting longer cells. This suggests that changes in tissue morphology observed in this experiment represent the "sum" of changes in the morphologies of all growing cells. The tendency for meristoderm cells to be longer in the highly weighted longitudinal kelps and wider in the highly weighted transverse kelps most likely reflects increased, or at least more unified, cell elongation (possibly leading to division) in the principal direction of tensile force (Biro et al. 1980), as predicted by (Gerard

1987). To verify this hypothesis, we need to observe axes of meristoderm cell divisions directly and examine whether these were altered by the application of tension.

2.5.3 *Nereocystis* is sensitive to the location of mechanical loading

My data indicate that only mechanical loading applied directly to actively growing meristematic tissue will evoke morphological plasticity in *Nereocystis* blades. This is consistent with my original hypothesis and my observations from the load direction experiment. The lack of response to a stimulus applied far from the tissue region where the response is generated reinforces my previously discussed conclusion that a long-distance signaling mechanism most likely does not mediate the effect of mechanical loading on kelp blade morphology.

If mechanical loads must be imposed directly on growing tissue by drag, then there must be tissue located distal to that growing tissue for drag to act upon. In other words, the mechanism being utilized by *Nereocystis* to facilitate its flow-induced plasticity may be reliant on intercalary meristems located at the proximal ends of blades. This may explain, in part, why morphological plasticity across hydrodynamic gradients in seaweeds has been best described and most consistently observed in kelps up to this point (e.g. Druehl and Kemp 1982, Gerard 1987, Buck and Buchholz 2005, Fowler-Walker et al. 2006, Koehl et al. 2008), as this whole group prominently exhibits intercalary growth (Fritsch 1923, Graham et al. 2017). It could also potentially explain why it has been historically difficult to convincingly demonstrate plasticity in seaweeds that rely on apical meristems, such as red algae (Floc'h 1969, Shaughnessy 2004) and the brown alga *Fucus* (Sideman and Mathieson 1983, 1985, Blanchette 1997). In a situation where growth was entirely apical, drag simply could not be imposed directly on the growing tissue. If plasticity were observed in spite of this, I would infer that some form of long-distance

signaling mechanism would be needed to communicate the perception of the stimulus from more proximal tissue to the growing meristem.

2.6 Conclusions

In summary, I conducted three experiments to investigate how morphological plasticity in response to mechanical loading in *Nereocystis* blades was affected by the (1) magnitude, (2) direction, and (3) location of the applied force. I found that kelp blades subjected to a gradient of tensile force grew progressively narrower, longer, less ruffled, and, unexpectedly, heavier as the magnitude of loading increased; the linear reaction norms observed suggests that *Nereocystis* is equally well-adapted for all flow environments tested. I found that when high tensile force was applied transversely across blade tissue, the response seen under high longitudinal tension was rotated 90°. This indicates that the entire set of physiological mechanisms that facilitate the plasticity most likely occurs within the same individual meristematic cells, suggesting against the involvement of a long-distance signaling mechanism. Furthermore, as the average morphology of a blade's meristoderm cells paralleled that of the tissue in this experiment, I infer that changes in blade morphology probably reflect altered cell elongation patterns. Finally, I found that the growth response to tension only occurred when mechanical loading was applied directly to the meristematic tissue, reinforcing that there is probably no long-distance signaling involved and indicating that response to hydrodynamic forces likely requires an intercalary meristem to facilitate mechanoperception. This study provides information on the evolutionary relationship between *Nereocystis* and water flow while lending insight into cellular mechanisms that might facilitate morphological plasticity in kelps.

3. Auxins as regulators of growth and morphogenesis in the kelp *Nereocystis luetkeana*

3.1 Synopsis

Some kelps exhibit morphological plasticity across hydrodynamic gradients, developing broad blades in slow flow and narrow blades in fast flow. While the functional significance of this plasticity as an adaptation to reduce drag has been relatively closely examined, little is known about the developmental mechanisms that underly the changes in blade morphology. In this chapter, I examined whether kelp plasticity could potentially be mediated by auxins by culturing blade tissue disks of the kelp *Nereocystis luetkeana* in the presence of a range of concentrations of the auxin NAA and measuring changes in disk size and morphology. I found that an NAA concentration of 10^{-5} M caused tissue disks to grow longer and narrower, but not heavier, than control disks. This indicates that auxins can have morphogenic effects on *Nereocystis*. Auxins may cause these effects by increasing the extensibility of kelp cell walls, thereby facilitating cell elongation, much like how they function in land plants. The anisotropic growth observed may be due to pre-existing anisotropy in the material properties of the kelp meristematic cell walls or anisotropic effects of auxin on different wall faces. The effects of the 10^{-5} M NAA treatment on *Nereocystis* blade tissue were strikingly similar to those of drag-induced tensile stress. While this is not direct evidence that auxins are involved in regulating morphological plasticity in response to hydrodynamic forcing, the tantalizing similarity between the responses to auxin and mechanical loading suggest that auxin signaling may play a role in kelp plasticity. This hypothesis deserves further study.

3.2 Introduction

Several species of kelps show pronounced morphological plasticity across hydrodynamic gradients (e.g. Gerard and Mann 1979, Druehl and Kemp 1982, Gerard 1987, Fowler-Walker et al. 2006, Koehl et al. 2008). These species generally develop broad blades when grown in wave- and current-sheltered environments and narrow blades when grown in wave- or current-exposed environments (e.g. Gerard and Mann 1979, Druehl and Kemp 1982, Gerard 1987, Fowler-Walker et al. 2006, Koehl et al. 2008). Individuals will also rapidly adjust blade morphologies when the ambient flow velocity changes (e.g. Gerard and Mann 1979, Druehl and Kemp 1982, Gerard 1987, Fowler-Walker et al. 2006, Koehl et al. 2008). This plasticity is generally interpreted as an adaptation to the hydrodynamic environment, permitting these kelps to minimize the amount of drag they experience in flow while maximizing productivity (Koehl and Alberte 1988).

While morphological plasticity across hydrodynamic gradients in kelps has been relatively well studied from a functional perspective (Koehl and Alberte 1988, Johnson and Koehl 1994, Buck and Buchholz 2005, Hurd and Pilditch 2011), a great deal remains unknown about how it occurs at a developmental level. Research on the developmental basis of kelp plasticity has largely taken place in the bull kelp, *Nereocystis luetkeana*. In this species, changes in blade morphology are mediated by mechanical loading imposed by drag, as demonstrated by experiments that have induced morphological changes in blades simply by hanging weights from growing tissue (Koehl et al. 2008, Coleman and Martone 2020, Ch. 2). The transition from broad to narrow blade shapes occurs as a result of growth being reallocated from tissue widening into tissue elongation in response to longitudinally-oriented tension (Coleman and Martone 2020, Ch. 2) and ruffles form in slow flow when blade margins elongate more quickly than blade midlines

(Koehl et al. 2008). Furthermore, the entire developmental pathway facilitating plasticity in *Nereocystis* takes place over short distances and is sensitive to the direction in which mechanical stimulation is applied (Coleman and Martone 2020, Ch. 2). However, little information is available about what exact signaling mechanisms or subcellular processes might be occurring to allow *Nereocystis* and other kelps to translate mechanical stimulation into changes in growth.

Regulation of growth and development in land plants is intimately dependent upon the activity of a wide variety of hormones (Taiz and Zeiger 2010). Among the most abundant and functionally diverse of these hormones are auxins, which play roles in virtually all aspects of plant development (Taiz and Zeiger 2010). Auxins are well known for their ability to promote plant cell elongation (reviewed in Majda and Robert 2018), although, like other hormones, their exact effect depends on their concentration and the type of tissue they are acting on (Taiz and Zeiger 2010). For example, the most abundant and functionally significant plant auxin, indole-3-acetic acid (IAA), has detectable effects on *Avena* (oat) coleoptile tissue elongation at concentrations lower than 10^{-6} M and will increase growth rates up until a concentration of about 10^{-5} M (Taiz and Zeiger 2010; Fig. 3.1). Above this concentration, the addition of more IAA becomes gradually less effective until it eventually starts inhibiting growth at a concentration between 10^{-4} and 10^{-3} M.

The influence of auxins on plant cell elongation is rooted in their ability to both induce changes in the material properties of plant cell walls and increase water uptake by the cell (reviewed in Cosgrove 2005, Majda and Robert 2018). Auxins induce localized decreases in pH that cause increases in the extensibility of plant cell walls (Rayle and Cleland 1992, Majda and Robert 2018), which, when combined with maintenance of turgor pressure, results in expansion of the cell (reviewed in Cosgrove 2005, Majda and Robert 2018). This expansion becomes

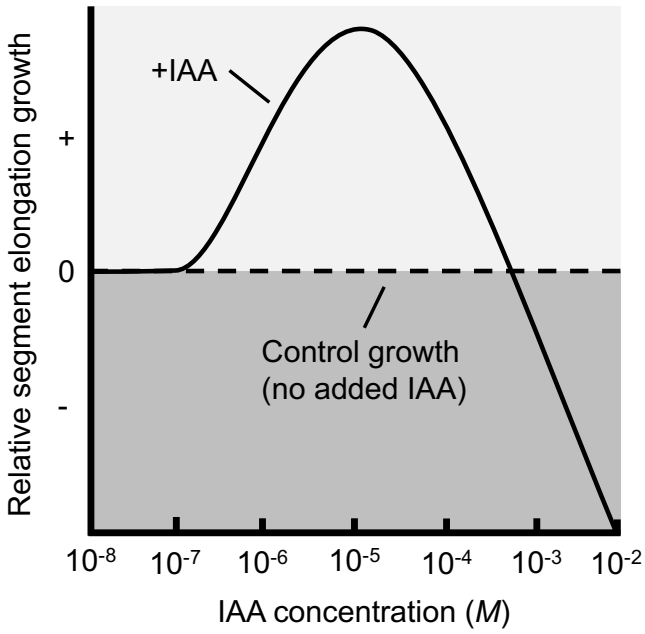

Fig. 3.1. A typical dose-response curve for IAA-induced elongation in pea stem or oat coleoptile sections; reproduced from Taiz and Zeiger (2010).

anisotropic when different parts of the cell wall are unequally extensible, which occurs primarily as a result of localized variation in the organization of cellulose microfibrils (reviewed in Baskin 2005, Wolf et al. 2012). The phenomenon of wall loosening induced by low pH leading to cell expansion is referred to as acid growth (Rayle and Cleland 1992, Majda and Robert 2018).

Plants possess a specialized auxin transport system that allows them to unidirectionally transport auxin between individual cells and throughout tissues (Taiz and Zeiger 2010). This polar transport of auxin gives plants a great deal of control over local concentrations, and therefore effects, of auxin in different organs and tissue types (Taiz and Zeiger 2010, Chen and Baluška 2013). Auxin is the only plant hormone that can be transported in a polarized manner (Taiz and Zeiger 2010). Evidence exists that plants can use the auxin transport system to alter auxin distribution in response to mechanical stimulation (Mitchell 1977, Erner and Jaffe 1982,

Hamant et al. 2008, Heisler et al. 2010, Sampathkumar et al. 2014b), indicating that auxins play important roles in mediating plant responses to mechanical signals.

While auxins are typically thought of as plant hormones, they also occur naturally in kelps (Hart 1982, Kai et al. 2006, Li et al. 2007), including *Nereocystis* (Van Overbeek 1940). Experimental addition of auxin has also been shown to increase the rates of tip elongation in the filamentous brown alga *Ectocarpus* (Rabillé et al. 2019) and blade elongation in the kelps *Alaria esculenta* (Buggeln and Bal 1977) and *Saccharina japonica* (Kai et al. 2006) along similar dose-response curves to those observed in plants. There is also evidence that auxin achieves this effect by increasing cell wall extensibility, much like it does in plants (Rabillé et al. 2019). Unfortunately, past studies that applied auxins to brown algae only measured changes in cell/tissue length, and so it is unknown whether morphological effects of auxin on brown algae might be more complex.

Koehl et al. (2008) proposed that, if hormones actually play central roles in regulating growth and development in kelps like they do in land plants, they could be involved in modifying growth patterns in response to mechanical stimulation. I suggest that auxins would be good candidate hormones for this role. It is possible, for instance, that mechanical loads imposed by drag could increase auxin concentrations in kelp meristematic tissue. This could result in increased rates of tissue growth, likely via increased expansion of the meristematic cells. Kelp tissues can show different degrees of extensibility in the longitudinal and transverse axes (Janot et al. 2012) and cellulose and alginate can be anisotropically distributed within brown algal cell walls (Terauchi et al. 2016). Given this, if *Nereocystis* cells had walls that were more extensible in the longitudinal axis than in the transverse axis, or if the auxin were able to increase extensibility of lateral wall faces more than longitudinal ones (possibly through anisotropy in

wall composition), then an increase in auxin may cause the kelp tissues to elongate more than widen. This would ultimately produce blades that were relatively long and narrow, like those typically observed in highly wave- or current-swept kelps (e.g. Gerard and Mann 1979, Johnson and Koehl 1994, Buck and Buchholz 2005, Fowler-Walker et al. 2006).

In order to investigate whether morphological plasticity across hydrodynamic gradients in kelps could potentially be regulated by auxin activity, I tested (1) how auxins affect kelp growth and morphology and (2) at what concentrations auxins are effective. In this chapter, I examined whether the application of exogenous auxin at two concentrations (10^{-5} M and 10^{-4} M) would have any morphogenic/growth effects on meristematic tissue of *Nereocystis luetkeana*. I hypothesized, based on Fig. 3.1 and the observations of Buggeln (1976), that both auxin treatments would increase the rate of tissue elongation compared to a control treatment, leading to the development of relatively long and narrow tissues, but that the 10^{-5} M treatment would have a stronger effect than the 10^{-4} M treatment.

3.3 Methods

A single blade was haphazardly selected and removed at its base from each of 30 mature *Nereocystis* sporophytes growing at Stanley Park near Brockton Point Lighthouse (49°17' 56."N, 123°07′00″ W) and brought to the University of British Columbia (UBC) on April 27, 2017. *Nereocystis* was chosen as the study kelp for this experiment due to it being the species with the best characterized growth/morphological response to water motion (Koehl et al. 2008, Coleman and Martone 2020, Supratya et al. 2020). Collected blades were stored in a sea table for up to 24 hours. A circular cork borer with a 1 cm diameter was used to remove three tissue disks from each collected blade. This cork borer was slightly flattened on one edge and the flat edge was

always oriented towards the distal end of the blade when a hole was punched. Tissue disks were punched out in a row from a region of tissue 10 cm from the blade base (Fig. 3.2), approximately the region of maximal active growth (Kain 1987, Koehl et al. 2008). The maximum length and width of each tissue disk was measured with vernier calipers to the nearest 0.1 mm; length was always oriented in the direction of the original longitudinal axis of the blade and width was oriented perpendicular to the length (Fig. 3.2). A Denver Instrument TP-214 microbalance was used to measure the wet mass of each tissue disk to the nearest 0.001 g; the surfaces of the tissue disks were wiped dry immediately before weighing.

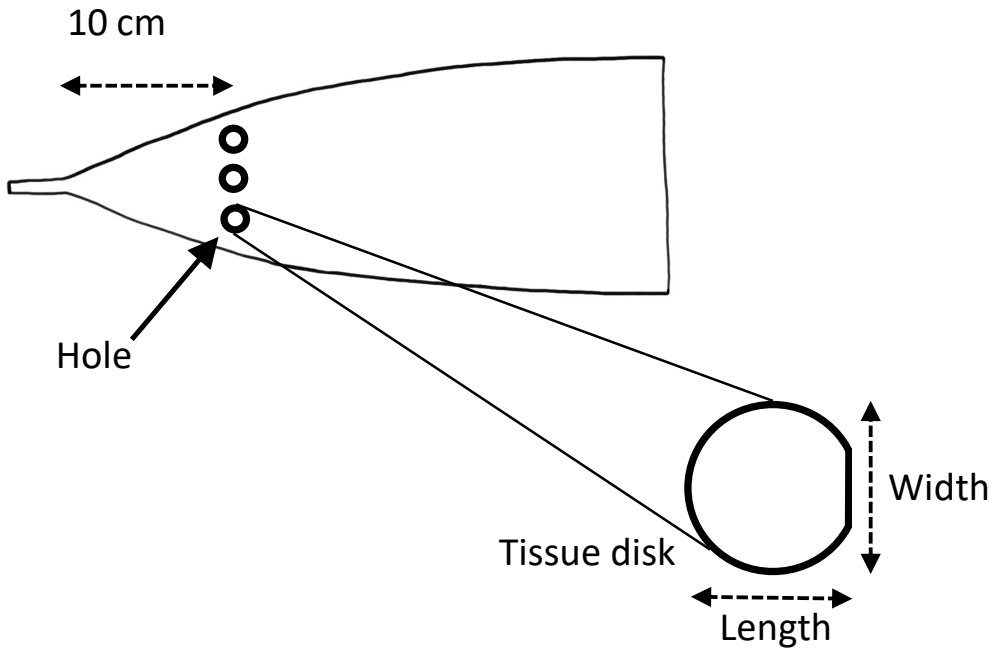

Fig. 3.2. *Nereocystis* blade tissue sampling and measurement method used in auxin experiment.

A single tissue disk was placed in each of 30 petri dishes containing half strength f/2 growth medium. The synthetic auxin 1-naphthaleneacetic acid (NAA; dissolved in 50% acetone) was added to 20 of those petri dishes such that 10 had a final NAA concentration of 10^{-5} M and the other 10 had a final NAA concentration of 10^{-4} M; the remaining 10 dishes acted as a control

treatment. NAA was chosen for use in this experiment over IAA because IAA is known to degrade when exposed to high light and salt concentrations (Yamakawa et al. 1979, Dunlap et al. 1986). The two experimental concentrations were chosen because they were hypothesized, based on existing data (Buggeln 1976, Kai et al. 2006), to be close to the concentration that would yield the greatest effect on growth. The 30 dishes were then placed at random positions on a shaker table housed in an incubator. The shaker rotated at a rate of 175 rpm in order to agitate the water in the dishes. The incubator maintained the tissue disks at a temperature of 10°C and exposed them to irradiance levels of 55-126 μmol m^{-2} s^{-1} (depending on the exact position of the dish in the chamber) on a 12:12 photoperiod. The tissue disks were left in place to grow for 72 hours.

At the end of the growth period, the tissue disks were removed from the incubator and the wet masses, lengths, and widths were measured for a second time. Principal components analysis (PCA) was used to identify the axes of maximal variation in the three measured morphological variables (expressed as percent change). PCA was used so that detection of experimental effects would not be confounded by correlation that existed among the morphological variables. Linear mixed effects models were used to analyze the effect of NAA concentration on the resulting principal components; sample blade was incorporated into each model as a random effect. All data analysis was performed in R (R Core Team 2021). Linear mixed effects models were performed using the lme() function from the nlme package (Pinheiro et al. 2020). Tukey-Kramer post-hoc tests were performed using the emmeans() function from the emmeans package (Lenth 2020). PCA was performed with the prcomp() function from the R stats package (R Core Team 2021); data were centered and scaled. Assumptions of normality were tested with Shapiro-Wilk tests performed using the shapiro.test() function from the R stats package (R Core Team 2021). Assumptions of homoscedasticity were tested with F-tests using the var.test() function from the

R stats package (R Core Team 2021). Assumptions of independence of residuals were tested with Durbin-Watson tests using the durbinWatsonTest() function from the car package (Fox and Weisberg 2019). No analyzed data were found to violate statistical assumptions.

3.4 Results

The first principal component (PC1) was strongly negatively correlated with mass (ΔM_D), length (ΔL_D), and width (ΔW_D) and was therefore interpreted as a metric of overall growth. The second principal component (PC2) was strongly negatively correlated with mass (ΔM_D), but strongly positively correlated with width (ΔW_D). The third principal component (PC3) was strongly positively correlated with length (ΔL_D), but strongly negatively correlated with width (ΔW_D) and was therefore interpreted as a metric of shape change. PC1 explained 68.9% of the variation in the data, PC2 explained 18.1% of the variation, and PC3 explained 12.9% of the variation (Table 3.1).

Table 3.1. Output of PCA performed for auxin application experiment.

Variable	Loadings		
	PC1	PC2	PC3
Change in disk wet mass (ΔM_D)	-0.550	-0.813	-0.190
Change in disk length (ΔL_D)	-0.600	0.226	0.768
Change in disk width (ΔW_D)	-0.581	0.536	-0.612
Proportion of variation	0.689	0.182	0.129

There was a near-significant effect of NAA concentration on growth (PC1; ANOVA, $F_{2,28}$=3.04, p=0.064) and a significant effect of NAA concentration on shape (PC3; ANOVA, $F_{2,28}$=3.45 p=0.046; Table 3.2; Fig. 3.3). There was no significant effect of NAA concentration on PC2 (ANOVA, $F_{2,28}$=0.258, p=0.77). The 10^{-5} M treatment disks grew significantly longer

and narrower than the control disks (Tukey, p=0.037). See Appendix B for raw morphological data.

Table 3.2. ANOVA tables for linear mixed effects models used to analyze principal component scores in auxin application experiment.

Response	numDF	denDF	F	p
PC1 (growth)	2	28	3.04	0.064
PC2	2	28	0.258	0.774
PC3 (elongation, -widening)	2	28	3.45	0.046

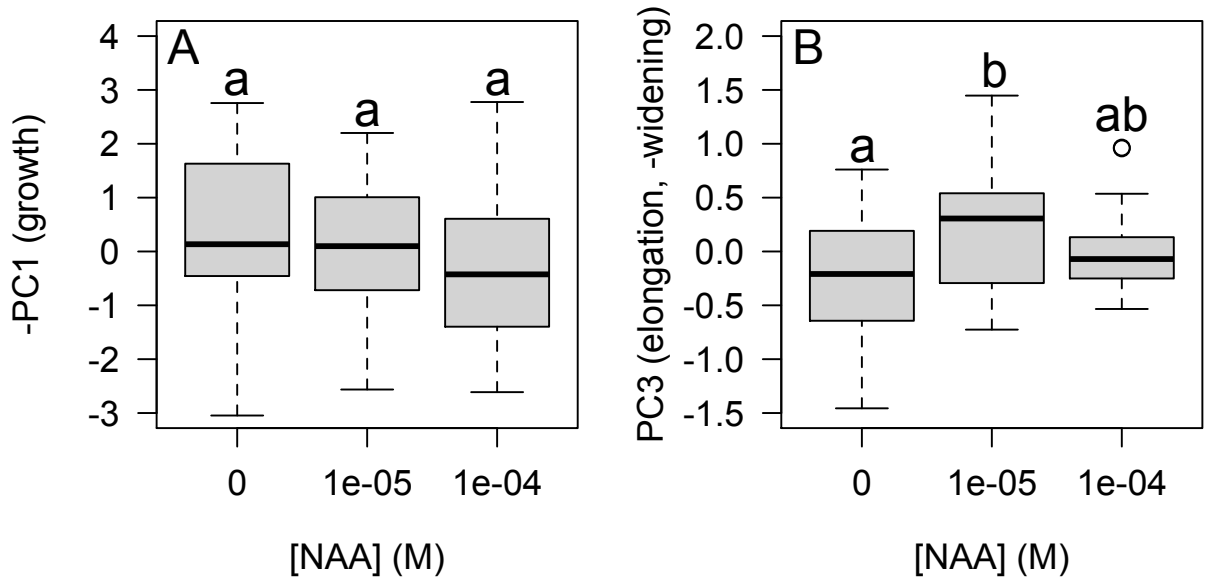

Fig. 3.3. Change in morphological variables of *Nereocystis* blade tissue disks expressed as functions of NAA concentration. Lowercase letters denote significantly different groups as indicated by a Tukey-Kramer post-hoc test.

3.5 Discussion

The results of this experiment indicate that auxins have morphogenic effects on *Nereocystis*. When *Nereocystis* meristematic tissues were grown in the presence of the auxin NAA at a concentration of 10^{-5} M, the tissues grew relatively long and narrow (with respect to the original longitudinal axis of the blade) compared to a control treatment. This was consistent

with the hypothesis that the addition of auxin would increase the rate of tissue elongation and results are comparable to those of past studies examining the effects of auxin application on kelp growth (Buggeln 1976, Kai et al. 2006). However, the decrease in widening and lack of change in overall growth observed indicate that the effect of auxin on kelp tissue is not to simply increase growth rates. The observed increase in tissue elongation likely reflects increased cell expansion in the longitudinal axis. In land plants, auxin mediates cell elongation by reducing the elastic modulus of cell wall and increasing water uptake by the cell to maintain turgor pressure, which results in expansion of the cell (reviewed in Cosgrove 2005, Majda and Robert 2018). The increased elongation observed in *Nereocystis* could possibly be driven by a similar mechanism. This hypothesis is further supported by evidence that auxins increase extensibility of cell walls in *Ectocarpus* (Rabillé et al. 2019). It has also been previously observed that brown algal cells are maintained under positive turgor (Kropf et al. 1995, Rabillé et al. 2019) and exhibit wall loosening prior to expansion (Hable and Kropf 1998).

In land plants, anisotropic growth of cells is facilitated by localized variation in the extensibility of the cell walls (reviewed in Baskin 2005, Wolf et al. 2012). With this in mind, the fact that the kelp tissues exposed to the 10^{-5} M auxin treatment elongated more than they widened may indicate that the meristematic cells of the kelp already had walls that were less extensible in the transverse axis than in the longitudinal axis. It could also indicate that the auxin treatment decreased the extensibility of the lateral wall faces more than it did in the than the longitudinal wall faces, possibly due to anisotropy in wall composition. Brown algae have been observed to show anisotropy in the cellulose and alginate distributions throughout their cell walls (Terauchi et al. 2016) as well as differences in extensibility of tissues depending on whether it is measured in the longitudinal or transverse axis (Janot et al. 2012). To examine more closely

whether anisotropy in wall properties and/or composition could facilitate the effects of auxin application observed here, a technique like atomic force microscopy (Tesson and Charrier 2014) could be used to measure the wall properties of kelp meristematic cells in different axes and see if they differ. This technique could also be used to directly measure whether the application of auxin affects kelp wall properties equally in all axes.

When meristematic tissues were cultured in the presence of NAA at a concentration of 10^{-4} M, they showed a trend towards decreased overall growth compared to the control treatment, although the effect was not statistically significant. This was inconsistent with my original hypothesis that concentrations of both 10^{-5} and 10^{-4} M would increase tissue elongation, but is consistent with the known effects of high concentrations of auxins on plant and kelp tissues (Buggeln 1976, Kai et al. 2006, Taiz and Zeiger 2010). This likely indicates that either the initial concentration of auxin in the kelp tissue was high and the addition of the 10^{-4} M NAA treatment increased the overall auxin concentration to inhibitory levels, or that *Nereocystis* displayed a low threshold of auxin concentration beyond which the hormones become inhibitory.

The increased elongation and reduced widening induced in *Nereocystis* blade tissue by the 10^{-5} M auxin treatment are remarkably similar to the effects produced in the same tissue by the application of mechanical loading (Coleman and Martone 2020, Ch. 2). While there is no direct evidence directly connecting the effects of auxin with those of mechanical loading, the striking similarity in the morphogenic effects of the two factors on *Nereocystis* raises interesting questions about how they may be linked. Given that increasing auxin concentrations can increase the extensibility of the cell wall in plants (Braybrook and Peaucelle 2013) and that mechanical stimulation can induce changes in auxin transport patterns and concentrations in plants (Mitchell 1977, Erner and Jaffe 1982, Hamant et al. 2008, Heisler et al. 2010, Sampathkumar et al. 2014b),

it may be possible that drag-induced tension on *Nereocystis* blades induces increases in auxin concentrations in the kelp's meristematic cells. This would then, theoretically, cause loosening of the cell walls, which would result in cell expansion (Cosgrove 2005, Braybrook and Peaucelle 2013, Majda and Robert 2018). The anisotropic growth observed under high mechanical loading could then indicate that the lateral wall faces are naturally more extensible than the longitudinal wall faces or that auxin has a disproportionately strong loosening effect on the lateral faces. Further exploration of the hypothesis that auxin mediates kelp plasticity in response to mechanical loading could proceed by measuring endogenous auxin concentrations in weighted and unweighted kelp tissue to see if the application of mechanical loads increases auxin concentrations. A sequenced kelp genome (e.g. Ye et al. 2015) could also be used to search for genes comparable to those known to interact with auxins, such as the PIN1 auxin efflux carrier found in plants. If such genes were identified, immunolabeling could be used to visually track how the movement of a PIN1-like protein might be influenced by mechanical loading, which could tell us whether kelps were actually modifying auxin transport patterns in response to mechanical stimulation.

3.6 Conclusions

In this chapter, I explored the possibility that auxins might play a role in regulating morphological plasticity across hydrodynamic gradients in kelps. Culturing disks of *Nereocystis* blade tissue in 10^{-5} M NAA, a synthetic auxin, caused the disks to grow longer and narrower, but not larger overall, compared to control disks, indicating that auxins can have morphogenic effects on *Nereocystis*. Auxins may work by increasing the extensibility of kelp cell walls, thereby facilitating cell elongation, much like how they function in land plants. The anisotropic

growth observed may be due to pre-existing anisotropy in the material properties of the kelp meristematic cell walls or anisotropic effects of auxin on different wall faces. The morphological changes observed in *Nereocystis* tissue when cultured in 10^{-5} M NAA are strikingly similar to those observed when the same tissues are subjected to sustained tensile force. While this is not direct evidence that auxins are involved in regulating morphological plasticity in response to drag, similar responses to auxin and mechanical loading are tantalizingly suggestive that auxin signaling may play a role in kelp plasticity and deserve further study.

4. The potential role of Ca^{2+} signaling in mechanoperception in the kelp *Macrocystis pyrifera*

4.1 Synopsis

Some species of kelps show conspicuous morphological plasticity in response to changes in their flow environment. Individuals will often grow broad, productivity-enhancing blades in slow flow and narrow, drag-reducing blades in fast flow. The biomechanical consequences of this phenomenon are relatively well understood, but very little is known about how, developmentally speaking, these kelps undergo such radical shape changes. In this chapter, I conducted two experiments to investigate whether stretch activated Ca^{2+} channels might play a role in the ability of kelps to perceive the drag-induced tensile stimuli that mediate the plasticity. I found that culturing *Macrocystis pyrifera* sporophytes in artificial seawater with the concentration of Ca^{2+} reduced 50% from normal had no effect on the kelp's morphological response to mechanical loading, indicating that either (1) stretch activated Ca^{2+} channels are not involved in kelp mechanoperception, (2) a 50% reduction in Ca^{2+} concentration was insufficient to inhibit mechanoperception, or (3) Ca^{2+} signaling is involved in mechanoperception but the ions are derived from internal stores. Any reduction in Ca^{2+} concentration greater than 50% resulted in total and sometimes rapid mortality of kelps. This is likely due to a loss of Ca^{2+} from the kelp cell walls, which would result in wall weakening, cellular expansion, and ultimately lysis. An observed increase in size of kelps subjected to 50% reductions in Ca^{2+} is also likely the result of non-lethal cellular swelling induced by this mechanism.

4.2 Introduction

Brown algae of the order Laminariales, better known as kelps, sometimes show a remarkable ability to change their thallus shape depending on their hydrodynamic environment (e.g. Gerard and Mann 1979, Druehl and Kemp 1982, Buck and Buchholz 2005, Fowler-Walker et al. 2006, Koehl et al. 2008). Species such as *Nereocystis luetkeana* (Koehl and Alberte 1988, Johnson and Koehl 1994), and *Macrocystis pyrifera* (Druehl 1978, Druehl and Kemp 1982) will grow blades that are narrow and flat when ambient flow speeds are fast. But, when the same individuals are transplanted to areas with slow ambient flow, the blades will rapidly develop broad and sometimes undulate shapes (Druehl and Kemp 1982, Koehl et al. 2008). The narrow blades found in fast flow tend to compress into clumps as water moves past them, which reduces the amount of drag experienced by the kelp, but also causes blades to shade each other, which negatively impacts productivity (Koehl and Alberte 1988, Johnson and Koehl 1994). Broad blades, conversely, tend to spread out and flap in flow, which minimizes the amount that blades shade each other at the cost of increasing drag (Koehl and Alberte 1988). Collectively, this morphological plasticity has been interpreted by phycologists as an adaptation that allows kelps like *Nereocystis* and *Macrocystis* to continuously reduce the amount of drag they experience while increasing productivity (Koehl and Alberte 1988).

Most research on the morphological plasticity of kelps has been focused on its functional significance (e.g. Koehl and Alberte 1988, Johnson and Koehl 1994, Hurd and Pilditch 2011) and relatively little is known about the developmental processes that underly it. Experiments in which weights were hung from blades of *Nereocystis luetkeana* (Koehl et al. 2008, Coleman and Martone 2020, Ch. 2) and *Saccharina latissima* (Gerard 1987) have revealed that the plasticity is regulated by mechanical loading that would naturally be imposed on kelps by drag. These

experiments have also indicated that drag-induced shifts from broad to narrow blade morphologies are the result of the kelps reallocating growth from blade widening into blade elongation (Gerard 1987, Coleman and Martone 2020, Ch. 2). However, practically nothing is known about how kelps perceive the mechanical cues that induce the plasticity, or how they translate those cues into changes in spatial patterns of growth.

A critical component of any form of adaptive phenotypic plasticity is a mechanism of cue perception (Smith 1990, Getty 1996, Schlichting and Smith 2002). While it is not known how kelps perceive mechanical cues, such mechanisms are comparatively well understood in land plants. Thigmomorphogenesis is a term used to describe morphogenic or developmental effects induced in plants by mechanical stimulation (Jaffe 1973). Common examples of this phenomenon are decreases in elongation and increases in radial expansion of plant stems induced by stem bending (Beryl and Mitchell 1977, Biddington 1986, Garner and Langton 1997, Braam 2005, Chehab et al. 2008). Thigmomorphogenesis has generally been interpreted as an adaptive phenomenon to help plants resist mechanical perturbations such as wind (Biddington 1986, Telewski and Jaffe 1986, Braam 2005, Chehab et al. 2008).

In addition to the ultimate developmental effects of thigmomorphogenesis, it has been repeatedly observed that mechanical stimuli can induce immediate increases in both cytosolic Ca^{2+} concentrations (Toriyama and Jaffe 1972, Knight et al. 1992, Trewavas and Knight 1994) and expression levels of genes encoding proteins that interact with Ca^{2+}, such as calmodulins (e.g. Braam and Davis 1990, Braam 1992, Lee et al. 2005) in plant cells. The observed increases in cytosolic Ca^{2+} are often thought to reflect activity of stretch-activated Ca^{2+} channels embedded in plasma membranes (Ding and Pickard 1993, Braam 2005, Telewski 2006, Kaneko et al. 2009, Monshausen and Gilroy 2009, Monshausen and Haswell 2013), although evidence also exists

that mechanically-induced Ca^2 fluxes can be derived from internal cellular stores (Knight et al. 1992). Furthermore, it has been shown that both Ca^{2+} chelators and calmodulin antagonists can reduce thigmomorphogenic effects of touch stimuli (Jones and Mitchell 1989). Collectively, these lines of evidence suggest that Ca^{2+} signaling plays key roles in plant mechanoperception and in facilitating thigmomorphogenesis. A simple model for plant mechanoperception has been proposed as follows: (1) mechanical stimuli deform plant cell walls, which stretches plasma membranes, (2) stretch-activated Ca^{2+} channels in the plasma membrane open, flooding the cytosol with Ca^{2+}, (3) Ca^{2+} interacts with calmodulins or calmodulin-like proteins, (4) calmodulins interact with other molecules in order to produce the ultimate response to the mechanical stimulus, which may involve changes in gene expression (Jaffe et al. 2002, Monshausen and Gilroy 2009, Monshausen and Haswell 2013).

Although they are distantly related within the tree of eukaryotes (Keeling and Burki 2019), kelps and land plants are ecologically similar (Steneck et al. 2002) and show remarkable morphological and anatomical convergence (Drobnitch et al. 2015, Starko and Martone 2016a). It is plausible that they could have evolved a similar reliance on Ca^{2+} signaling for mechanosensing, especially given how common Ca^{2+} signaling is throughout the tree of life (Batiza et al. 1996, Berridge et al. 2000, Jaffe et al. 2002, Clapham 2007). If kelps were to utilize Ca^{2+} signaling in a similar way to land plants for sensing the mechanical stimuli that induce morphological plasticity across flow gradients, then I would hypothesize that tension imposed by drag first deforms the walls of the kelp's meristematic cells, triggering the opening of stretch activated Ca^{2+} channels, which are already known to exist in brown algae (Taylor et al. 1996, Verret et al. 2010). This would result in an influx of Ca^{2+} ions that would theoretically initiate an series of developmental processes that would ultimately result in meristematic cells tending to

grow and divide more in the longitudinal axis of the blade than in the transverse axis (Coleman and Martone 2020, Ch. 2). These developmental processes could include mediation by calmodulins or calmodulin-like proteins. Calmodulins are known to be required for the establishment of polarity in *Fucus serratus* zygotes (Love et al. 1997, Brownlee et al. 2001) and could be similarly necessary in determining the direction of growth and division in kelp meristematic cells. Active, Ca^{2+}-bound calmodulins could influence directions of cell growth by inducing changes in the expression of genes that affect the cytoskeleton or cell wall, or possibly by interacting with cytoskeletal or wall elements directly (Bouché et al. 2005, Perochon et al. 2011). For example, actin filaments, which are heavily involved in cell wall synthesis in brown algae (Katsaros et al. 2002, 2003, 2006), could possibly be influenced through the activity of calmodulins to build up certain faces of the cell walls in order to direct cell elongation (Baskin 2005, Wolf et al. 2012). Both the actin cytoskeleton (Kropf et al. 1989) and the cell wall (Kropf et al. 1988, Quatrano and Shaw 1997) are required to establish cell polarity in *Fucus* zygotes and are likely to be important for determining directions of growth and division in kelp meristematic cells, with or without the involvement of calmodulins.

To begin to examine whether a mechanism such as the one described above might facilitate kelp mechanoperception and morphological plasticity, I conducted two experiments to search for evidence that morphological plasticity in the kelp *Macrocystis pyrifera* was dependent upon the activity of plasma membrane-bound stretch activated Ca^{2+} channels, as these are one of the mechanistic features most often suggested to underly plant mechanoperception (Jaffe et al. 2002, Monshausen and Gilroy 2009, Monshausen and Haswell 2013). The first experiment, hereafter referred to as the "calcium exclusion experiment," investigated whether the kelp growth response to mechanical loading could be negated by removing all Ca^{2+} from the

environment. I hypothesized that this would completely inhibit or substantially reduce the magnitude of the growth response compared to a control. The second experiment, hereafter referred to as the "calcium gradient experiment," investigated whether there was any Ca^{2+} concentration lower than that of natural seawater that could affect kelp plasticity without causing mortality, as kelps subjected to calcium-free seawater in the calcium exclusion experiment did not survive. I hypothesized that there would be lower-than-normal Ca^{2+} concentrations that would weaken the kelp growth response to mechanical loading.

4.3 Methods

4.3.1 Calcium exclusion experiment

Macrocystis pyrifera sporophytes were cultured from spores by Monterey Bay Seaweeds in Moss Landing, California. Sporophytes were grown in tumble culture until they reached a length of 10-20 cm and were then shipped to the University of British Columbia. Upon arrival, sporophytes were stored in containers of natural seawater until they were prepared for experimental use. On February 20, 2021, 40 individual kelps were haphazardly selected; chosen kelps consisted of a single blade with no splits in progress and had intact stipes and holdfasts. Sample kelps were cut to a standard initial length of 5 cm as measured from the base of the blade and then photographed and weighed. The software ImageJ (Rasband 2021) was used to measure the length, width at 1 cm from the base, and surface area of all experimental blades (Fig. 4.1). Plastic clothespins were used to hang 10 kelps vertically by the holdfast in each of four 20.8 L aquaria such that kelps were suspended in mid-water (Fig. 4.2A). Two of the four aquaria were filled with MBL artificial seawater (Lyman and Fleming 1940, Cavanaugh 1975; formulation in Table 4.1), hereafter referred to as the "full Ca^{2+}" treatment, while the other two were filled with

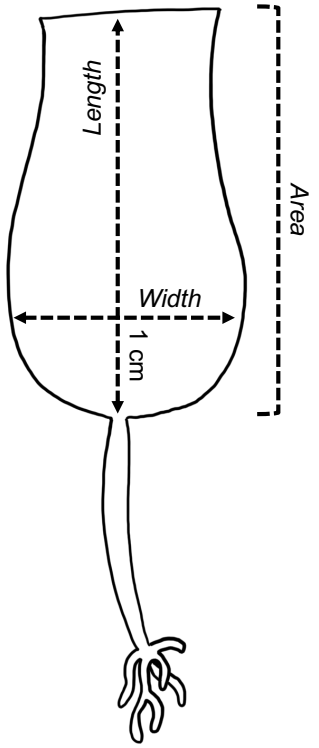

Fig. 4.1. Morphological measurements taken for *Macrocystis pyrifera* sporophytes.

Table 4.1. Artificial seawater recipes used in calcium exclusion experiment.

Ingredient	Concentration (mM)	
	MBL (full Ca)	Ca-free MBL
NaCl	423	436.71
$CaCl_2$*$2H_2O$	**9.27**	**0**
KCl	9	9
$MgCl_2$*$6H_2O$	22.94	22.94
$MgSO_4$*$7H_2O$	25.5	25.5
$NaHCO_3$	2.14	2.14

calcium-free MBL artificial seawater (Cavanaugh 1975; Table 4.1), hereafter referred to as the "Ca^{2+}-free" treatment. All kelps had a second clothespin attached to their distal ends, but a random five individuals from each of the four tanks had a 0.56 N weight attached to that clothespin (Fig. 4.2A). Individuals that bore this additional weight are hereafter referred to as belonging to the "high weight" treatment, while those that did not are hereafter referred to as

belonging to the "low weight" treatment. The overall experiment was a factorial design with weight and calcium treatments and two experimental blocks to control for tank effects (Fig. 4.2B). Kelps were maintained in the aquaria at a temperature of 10°C and a salinity of 34 ppt. They were exposed to ~50 μmol m^{-2} s^{-1} of photons on a 12:12 cycle. Nutrients were added to the artificial seawater such that the kelps were cultured in the equivalent of f/2 growth medium. Small submersible pumps were placed in the tanks to gently circulate the water. The kelps were left in place to grow for seven days.

At the end of the growth period, blade length, width, area, and wet mass were re-measured for all surviving kelps. Principal components analysis (PCA) was used to identify the axes of maximal variation in four measured morphological variables (expressed as percent change). PCA was used so that detection of experimental effects on individual morphological variables would not be confounded by the fact that some of the morphological variables were correlated. ANOVA was used to analyze the effect of weight and tank on the resulting principal component scores. The Kaiser method was used to determine which principal components to analyze (Kaiser 1960). The effect of calcium concentration was not analyzed, as no kelps immersed in calcium-free seawater survived the experiment. Tank was incorporated into the ANOVAs as a fixed effect instead of a random effect due to the low number of blocks (Crawley 2002, Hodges 2013). All data analysis was performed in R (R Core Team 2021). PCA was performed with the prcomp() function from the R stats package (R Core Team 2021); data were centered and scaled. ANOVA was performed using the lm() and anova() functions from the R stats package package (R Core Team 2021). Tukey-Kramer post-hoc tests were performed using the emmeans() function from the emmeans package (Lenth 2020). Assumptions of normality were tested with Shapiro-Wilk tests performed using the shapiro.test() function from the R stats

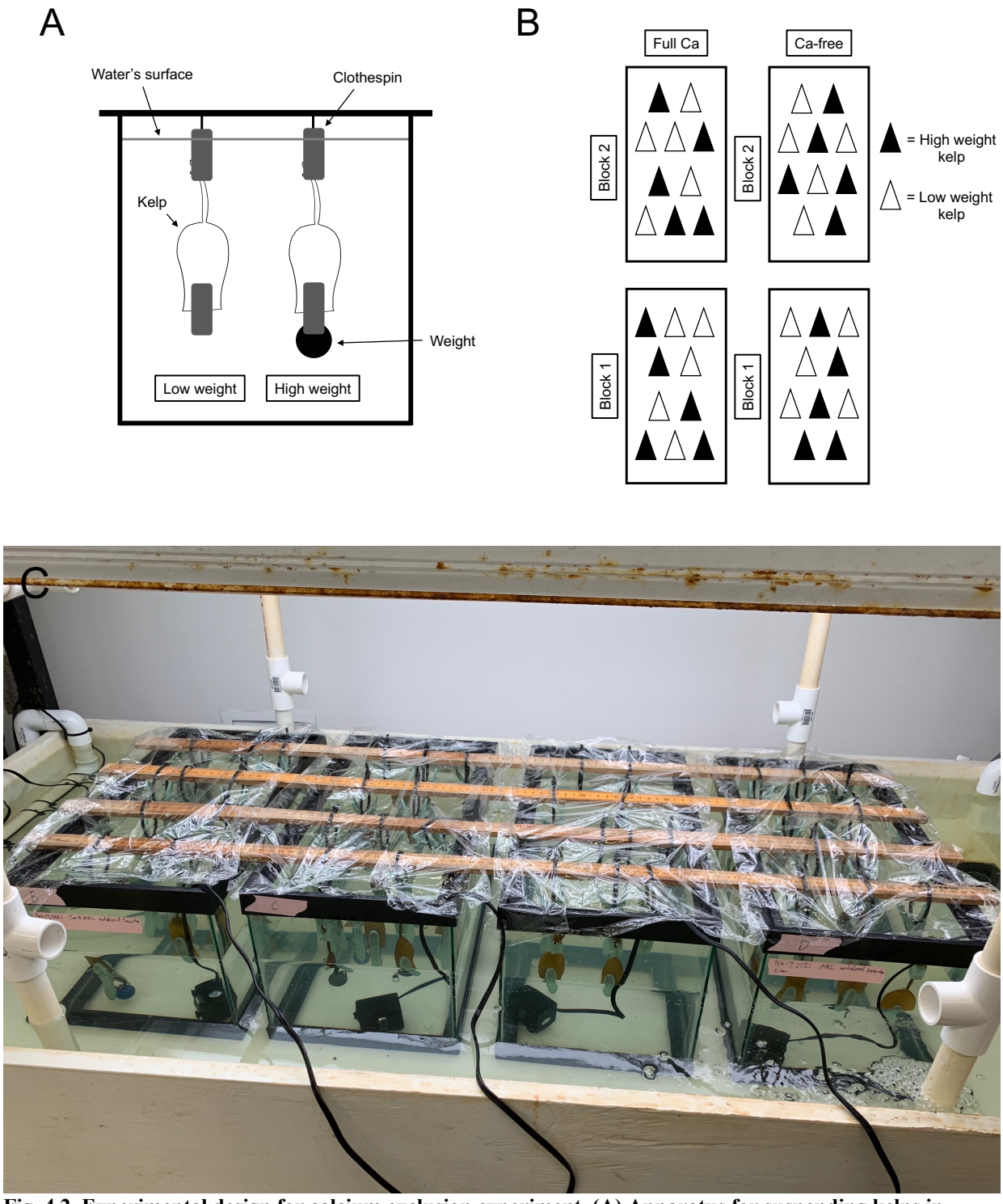

Fig. 4.2. Experimental design for calcium exclusion experiment. (A) Apparatus for suspending kelps in tanks. (B) Organization of sample kelps from different weight treatments within Ca^{2+} treatments and experimental blocks. (C) Photograph of experimental apparatus.

package (R Core Team 2021). Assumptions of homoscedasticity were tested with F-tests using the ols_test_f() function from the olsrr package (Hebbali 2020). No analyzed data were found to violate statistical assumptions.

4.3.2 Calcium gradient experiment

A second shipment of *Macrocystis pyrifera* sporophytes were sourced from Monterey Bay Seaweeds in Moss Landing, CA as described in 4.3.1. On March 7, 2021, 50 individual kelps were haphazardly selected and were prepared for experimentation and morphologically characterized using the methods described in 4.3.1. Ten kelps were suspended vertically by their holdfasts in each of five 20.8 L aquaria. One aquarium was filled with MBL artificial seawater (Lyman and Fleming 1940, Cavanaugh 1975, Table 4.1), hereafter referred to as the "full Ca^{2+}" treatment, while the other four aquaria were filled with MBL artificial seawater with 1/2, 1/4, 1/8, and 1/16 the amount of calcium called for by the standard MBL recipe (Table 4.2), hereafter referred to as the "1/2 Ca^{2+}", "1/4 Ca^{2+}", "1/8 Ca^{2+}", and "1/16 Ca^{2+}" treatments respectively. Five random individuals from each tank had a 0.56 N weight attached to their distal ends, as described in 4.3.1 (Fig. 4.2A); individuals that bore this additional weight define the "high weight" treatment, while those that did not bear weights define the "low weight" treatment. The experiment had a factorial design with weight and calcium treatments (Fig. 4.3). Kelps were maintained in the aquaria with the same set of culture conditions as those in 4.3.1. The kelps were left in place to grow for eleven days.

Table 4.2. Artificial seawater recipes used in calcium gradient experiment.

Ingredient	Concentration (mM)			
	1/2 Ca MBL	1/4 Ca MBL	1/8 Ca MBL	1/16 Ca MBL
NaCl	430.36	433.26	434.98	435.83
$CaCl_2$*2H_2O	**4.64**	**2.32**	**1.16**	**0.58**
KCl	9	9	9	9
$MgCl_2$*6H_2O	22.94	22.94	22.94	22.94
$MgSO_4$*7H_2O	25.5	25.5	25.5	25.5
$NaHCO_3$	2.14	2.14	2.14	2.14

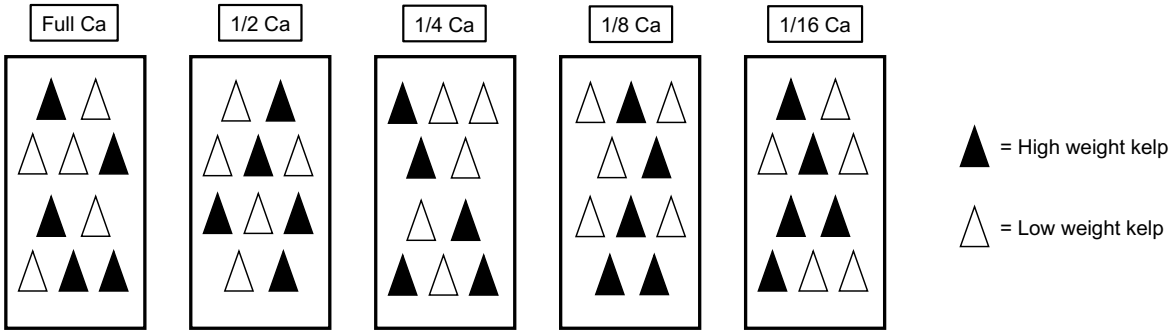

Fig. 4.3. Experimental design for calcium gradient experiment.

4.4 Results

4.4.1 Calcium exclusion experiment

All kelps subjected to the Ca^{2+}-free treatment died within minutes of the experiment beginning. Tissue surfaces became blistered, pigmentation faded, and tissues became soft and easily broken (Fig. 4.4). Remaining results for this section refer to the kelps from the full Ca^{2+} treatment only.

The first principal component (PC1) was positively correlated with all input variables, especially change in length and area, and was therefore interpreted as a metric of overall growth. The second principal component (PC2) was strongly positively correlated with change in length,

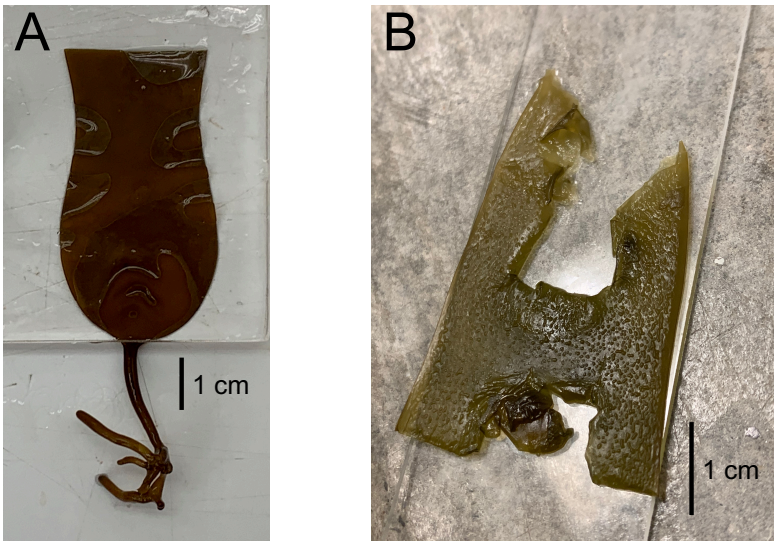

Fig. 4.4. *Macrocystis pyrifera* tissue (A) before and (B) after being immersed in Ca^{2+}-free seawater. Note the surface texture and loss of pigmentation in the kelp in image (B) compared to the one in image (A). Also note that the clothespins used for hanging the kelps have torn large piece of tissue from the kelp in image (B).

but strongly negatively correlated with change in width, and was therefore interpreted as a metric of shape change. PC1 explained 45.4% of the variation in the data and PC2 explained 30.0% of the variation (Table 4.3).

Table 4.3. Output of PCA performed for calcium exclusion experiment.

Variable	Loadings			
	PC1	PC2	PC3	PC4
Change in length	0.650	0.402	0.143	0.629
Change in width	0.125	-0.893	0.079	0.424
Change in area	0.700	-0.191	0.223	-0.652
Change in wet mass	0.269	-0.058	-0.961	-0.022
Proportion of variation	0.454	0.300	0.234	0.012

There were no significant effects of weight (ANOVA, $F_{1,16}$=1.23, p=0.283), or tank (ANOVA, $F_{1,16}$=2.50, p=0.134) on growth (PC1; Fig. 4.5A; Table 4.4). There was a significant

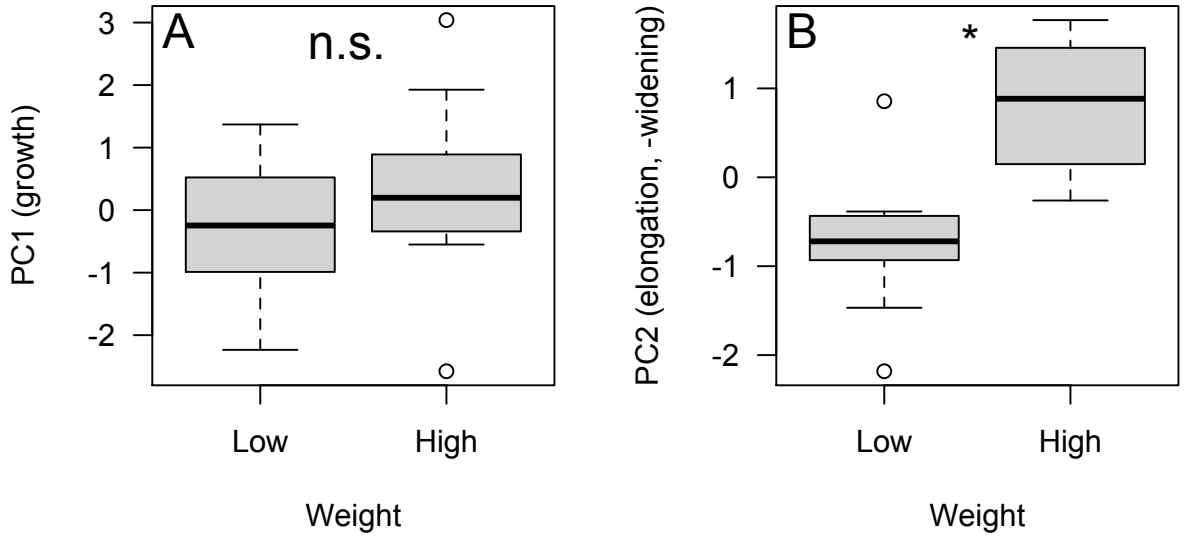

Fig. 4.5. Change in morphological variables of *Macrocystis pyrifera* from the full Ca^{2+} treatment group in calcium exclusion experiment expressed as functions of weight applied; "*" denotes significantly different group pairings as indicated by a Tukey-Kramer post-hoc test; "n.s." denotes a lack of significantly different groups as indicated by a Tukey-Kramer post-hoc test.

Table 4.4. ANOVA tables for calcium exclusion experiment.

Response	Explanatory	df	Sum Sq	Mean Sq	F	P
Principal component 1 (PC1; growth)	Weight	1	2.05	2.05	1.23	0.283
	Tank	1	4.14	4.14	2.50	0.114
	Residuals	16	26.5	1.66		
Principal component 2 (PC2; elongation, -widening)	Weight	1	12.0	12.0	21.4	**<0.001**
	Tank	1	0.58	0.583	1.04	0.323
	Residuals	16	8.97	0.561		

effect of weight (ANOVA, $F_{1,16}$=21.4, p<0.001), but no significant effect of tank (ANOVA, $F_{1,16}$=1.04, p=0.323), on shape (PC2; Fig. 4.5B; Table 4.4). The high weight groups grew significantly longer and narrower than the low weight groups (Tukey, p<0.001). See supplemental Fig. C1 for raw morphological data.

4.4.2 Calcium gradient experiment

No kelps from the 1/16, 1/8, or 1/4 Ca^{2+} treatments survived the experiment. These individuals exhibited the same symptoms as the individuals from the Ca^{2+}-free treatment in the calcium exclusion experiment. The rate of tissue deterioration was inversely correlated with calcium concentration, with kelps from the 1/16 Ca^{2+} treatments visibly dying within minutes of immersion, much like the Ca^{2+}-free kelps from the calcium exclusion experiment, and those from the 1/4 Ca^{2+} treatment deteriorating more slowly over several days. The remaining results for this section refer only to the kelps from the full Ca^{2+} and 1/2 Ca^{2+} treatments.

The first principal component (PC1) was positively correlated with all input variables and was interpreted as a metric of overall growth. The second principal component (PC2) was strongly positively correlated with change in length, but strongly negatively correlated with change in width, and was therefore interpreted as a metric of shape change. PC1 explained 51.1% of the variation in the data and PC2 explained 26.3% of the variation (Table 4.5).

Table 4.5. Output of PCA performed for calcium gradient experiment.

Variable	Loadings				
	PC1	PC2	PC3	PC4	PC5
Change in length	0.483	0.533	0.088	-0.189	-0.663
Change in width	0.194	-0.810	-0.128	-0.316	-0.437
Change in thickness	0.319	0.065	-0.900	0.276	0.086
Change in area	0.600	0.003	0.112	-0.515	0.602
Change in wet mass	0.517	-0.237	0.391	0.723	0.032
Proportion of variation	0.511	0.263	0.177	0.041	0.008

There were no significant effects of weight (ANOVA, $F_{1,16}$=1.08, p=0.314) or weight:calcium interactions (ANOVA, $F_{1,16}$=2.85, p=0.111) on growth (PC1), but there was a significant effect of calcium on growth (PC1; ANOVA, $F_{1,16}$=9.19, p=0.008; Fig. 4.6; Table 4.6). The high weight, full Ca^{2+} group showed significantly lower growth than both the low weight,

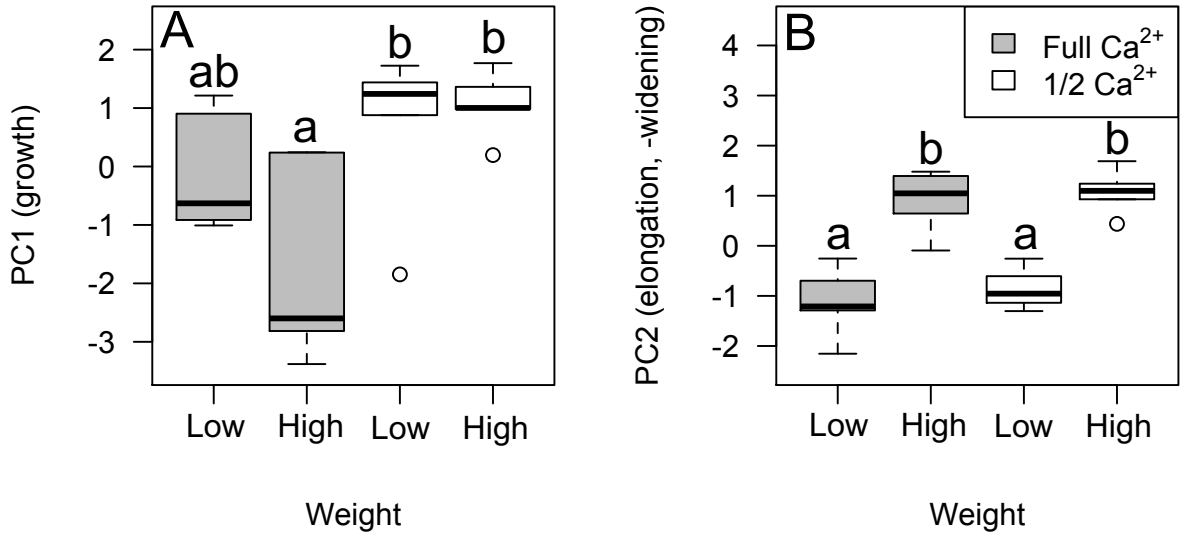

Fig. 4.6. Change in morphological characters of *Macrocystis pyrifera* sporophytes from full Ca^{2+} and 1/2 Ca^{2+} treatments used in calcium gradient experiment expressed as function of weight applied (low or high) and Ca^{2+} concentration. Lowercase letters indicate significantly different groups as indicated by a Tukey-Kramer post-hoc test.

Table 4.6. ANOVA tables for calcium gradient experiment.

Response	Explanatory	df	Sum Sq	Mean Sq	F	P
Principal component 1 (PC1; growth)	Weight	1	1.80	1.80	1.08	0.314
	Calcium	1	15.3	15.3	9.19	**0.008**
	Weight:Calcium	1	4.75	4.75	2.85	0.111
	Residuals	16	26.7	1.67		
Principal component 2 (PC2; elongation, -widening)	Weight	1	19.5	19.5	59.5	**<0.001**
	Calcium	1	0.257	0.257	0.787	0.388
	Weight:Calcium	1	0.009	0.009	0.028	0.870
	Residuals	16	5.23	0.327		

1/2 Ca^{2+} (Tukey, p=0.048) and high weight, 1/2 Ca^{2+} (Tukey, p=0.020) groups. There was a significant effect of weight (ANOVA, $F_{1,16}$=59.5, p<0.001), but no significant effects of calcium (ANOVA, $F_{1,16}$=0.787, p=0.388) or weight:calcium interactions (ANOVA, $F_{1,16}$=0.028, p=0.870), on shape (PC2). The high weight, full Ca^{2+} group grew significantly longer and narrower than both the low weight, full Ca^{2+} (Tukey, p<0.001) and low weight, 1/2 Ca^{2+} (Tukey, p<0.001) groups and the high weight, 1/2 Ca^{2+} group grew significantly longer and narrower

than both the low weight, full Ca^{2+} (Tukey, $p=0.001$) and low weight, 1/2 Ca^{2+} (Tukey, $p<0.001$) groups. See Fig. C2 for raw morphological data.

4.5 Discussion

The experiments discussed in this chapter did not reveal any detectable effect of reducing ambient Ca^{2+} concentrations on morphological plasticity in response to mechanical loading in the kelp *Macrocystis pyrifera*. Although the kelps in this experiment showed dramatic growth responses to continuous longitudinal tension that were consistent with those observed in *Nereocystis luetkeana* (Koehl et al. 2008, Coleman and Martone 2020, Ch. 2) and *Saccharina latissima* (Gerard 1987), reducing the Ca^{2+} concentration by half did not affect the magnitude of this response and reducing it by more than half proved lethal for the kelps. These results are contrary to my original hypothesis and indicate either that stretch activated Ca^{2+} channels are not involved in kelp mechanoperception or that a 50% reduction in ambient Ca^{2+} concentration was not enough to impede mechanically induced uptake of Ca^{2+} ions by the kelps. It is also possible that Ca^{2+} signaling is involved, but that the Ca^{2+} ions are derived from internal stores and do not rely upon transport of ions from the environment, as in Knight et al. (1992).

Perhaps the most striking result of these experiments was just how deadly substantial reductions in ambient Ca^{2+} were for the kelps. No kelps survived a greater than 50% reduction in Ca^{2+} concentration, with those subjected to reductions of greater than 75% visibly deteriorating within minutes of immersion. Kelps that were negatively affected by Ca^{2+} reduction showed physical symptoms similar to those observed in kelps suffering from hypoosmotic shock even though experimental salinities were maintained at a very typical 34 ppt. These symptoms included the formation of raised “blisters” on the tissue surface, loss of pigmentation, and tissues

becoming soft, limp, and easily broken. Use of the Ca^{2+} chelator EGTA to make Ca^{2+} in the environment unavailable does not generally induce such dramatic effects in land plant systems (e.g. Jones and Mitchell 1989, Nakagawa et al. 2007), but ambient Ca^{2+} concentrations of 10^{-5} M or less can damage plant cells (Picton and Steer 1983). Given that Ca^{2+} contributes to the structural integrity of brown algal cell walls by stabilizing alginate (Terauchi et al. 2016), it is likely that reducing environmental Ca^{2+} concentrations weakened the cell walls of the experimental kelps, causing the cells to swell when combined with turgor pressure. This assumes that kelp cells are under turgor like those of other brown algae (Kropf et al. 1995, Rabillé et al. 2019). In the case of the 1/2 Ca^{2+} treatment kelps from the calcium gradient experiment, such cellular swelling could explain the observed three-dimensional increase in thallus size compared to the full Ca^{2+} treatment kelps. However, when kelps were subjected to greater than 50% decreases in ambient Ca^{2+} concentration, cell wall integrity could have been fatally compromised, causing cells to swell to the point of bursting. This would greatly resemble the effect of a hypotonic solution on kelp cells.

The dramatic effects of reduced environmental Ca^{2+} concentrations on the kelps used in these experiments limited the utility of Ca^{2+} deprivation as a method for studying physiological roles of Ca^{2+} in kelps in this instance. How, then, could stretch activated Ca^{2+} channels be investigated given the tools currently available for studying kelp physiology? One possible approach would be to apply a mechanosensitive Ca^{2+} channel blocker, such as gadolinium or lanthanum (Knight et al. 1992, Klüsener et al. 1995, Knight 1999, White 2000), directly to kelp meristems to see if they inhibit plasticity, as these chemicals may interfere with the target channels without preventing the incorporation of Ca^{2+} into the kelp cell walls. This assumes that Ca^{2+} found in brown algal cell walls is derived at least partially from the environment and does

not come entirely from cytosolic Ca^{2+} that must first be selectively imported by channels. An alternative approach that does not explicitly investigate the activity of stretch activated channels, but still addresses a potential role for Ca^{2+} in regulating kelp plasticity, would be to see if kelps can still respond to mechanical loading in the presence of drugs that inhibit the activity of calmodulins (Jones and Mitchell 1989). Similarly, qPCR could be used to assess whether expression of calmodulin-like genes in kelps were upregulated during mechanical loading (Braam and Davis 1990).

4.6 Conclusions

In this chapter, I conducted two experiments to investigate whether morphological plasticity in response to mechanical loading in the kelp *Macrocystis pyrifera* could be partially or completely inhibited by reducing the ambient concentration of Ca^{2+}. I found that a 50% reduction in Ca^{2+} concentration had no effect on the kelp's response to tension, which suggests either that (1) stretch activated Ca^{2+} channels are not involved in kelp mechanoperception, (2) a 50% reduction in Ca^{2+} is insufficient to impede kelp plasticity, or (3) Ca^{2+} signaling is occurring, but the ions are derived from internal stores instead of from the environment. Kelps subjected to 50% Ca^{2+} concentrations also grew relatively large, likely reflecting swelling of cells as walls were destabilized. Greater than 50% reductions in Ca^{2+} concentration resulted in total, and in some cases, extremely swift mortality of kelps. Removal of Ca^{2+} from the environment likely caused loss of Ca^{2+} from the kelp cell walls, which would cause the walls to weaken and the cells to swell. When only 50% of Ca^{2+} was removed, this cellular swelling might merely have caused the kelps to increase in size, but when more than 50% of Ca^{2+} was removed, the kelp cells could have swollen to the point of lysis, resulting in mortality of the kelp. While removal of Ca^{2+} from

the environment was not a perfect method for studying the role of Ca^{2+} signaling in kelp mechanoperception, this line of inquiry could be expanded through experimentation with drugs that specifically inhibit Ca^{2+} channels or calmodulins, or through studies examining how mechanical loading affects the expression of genes coding for calmodulins or calmodulin-like proteins in kelps.

5. Grow with the flow: prevalence of phenotypic plasticity across hydrodynamic gradients in seaweeds

5.1 Synopsis

It is often said in phycology that seaweeds are highly phenotypically plastic across a range of environmental gradients, including those of hydrodynamic forcing. While many macroalgae show intraspecific phenotypic variation across gradients of water motion, researchers examining such variation often fail to test whether it is truly due to plasticity. In this chapter, I considered biomechanical and developmental mechanisms that might facilitate adaptive phenotypic plasticity across hydrodynamic gradients in seaweeds in order to make predictions about when such plasticity should be possible. I hypothesized that plasticity should be possible in any seaweed at slow flow velocities, where flow would be sensed chemically through boundary layers, but that it should only be possible in seaweeds with intercalary or diffuse growth at fast flow velocities, where flow would be sensed mechanically through drag. I tested these hypotheses by conducting a literature review to see how often phenotypic variation and plasticity have been observed to occur in seaweeds across hydrodynamic gradients. I found that phenotypic variation was well documented in brown algae but not well documented in red and green algae. Only 35% of all instances of variation were examined to see whether they might be due to phenotypic plasticity. Verifiable plasticity across hydrodynamic gradients was found to be well documented in brown algae, but not well documented in red and green algae. The data yielded mixed support my hypotheses with respect to the effects of flow velocity and growth mode on the occurrence of plasticity. The vast majority of cases of plasticity occurred in brown algae with intercalary meristems at a high range of flow velocities, but this could have been driven by the greater number of studies in brown algae than in red and green algae. There were also five

observations of plasticity occurring in seaweeds with apical meristems at high flow speeds; these observations merit further study. Overall, more research is needed to clarify the ability of red and green algae to respond to changes in flow, but phycologists should be aware that assumptions about phenotypic plasticity to flow in red and green algae are based on very little evidence.

5.2 Introduction

It is commonly said in phycology that seaweeds are highly phenotypically plastic across a range of environmental gradients (e.g. Kalvas and Kautsky 1993, Blanchette et al. 2002, Duggins et al. 2003, Miller et al. 2011, Díaz-Tapia et al. 2020), including those of hydrodynamic forcing. Indeed, many marine macroalgae have been observed to show conspicuous patterns of intraspecific phenotypic variability across gradients of water motion. Patterns commonly associated with increased wave or current exposure include the adoption of narrower, "streamlined" morphologies (Koehl and Alberte 1988, Armstrong 1989, Blanchette et al. 2002, Duggins et al. 2003, Buck and Buchholz 2005), reduction in thallus size (e.g. Blanchette 1997, Wolcott 2007), fortification of support tissues (Armstrong 1987, Johnson and Koehl 1994, Blanchette et al. 2002, Kitzes and Denny 2005), and increasing attachment strength (Jackelman and Bolton 1990, Kawamata 2001). These patterns have been largely interpreted by phycologists as adaptive phenomena facilitating increased endurance of seaweeds to increasing water motion through either drag reduction or increased tolerance to breakage or detachment (Armstrong 1987, Koehl and Alberte 1988, Blanchette et al. 2002, Wolcott 2007, Starko and Martone 2016b).

However, phenotypic variation is not necessarily indicative of phenotypic plasticity. Phenotypic plasticity specifically refers to trait variation induced by the environment (Stearns 1989). It may be either adaptive or not (Smith-Gill 1983, Stearns 1989, Padilla and Adolph 1996,

Ghalambor et al. 2007), and it may result from either active facilitation by the organism (e.g. a developmental program triggered by an environmental cue; Krueger and Dodson 1981, Smith-Gill 1983, Harvell 1984) or direct environmental intervention (e.g. physical damage caused by external factors; Smith-Gill 1983, Blanchette 1997, Wolcott 2007). Phenotypic plasticity should be contrasted with genetic differentiation, genetically fixed differences between individuals or populations that do not change with the environment (Alpert and Simms 2002). When such differences have a positive effect on an organism's fitness in a specific environment, it can be termed local adaptation (Kawecki and Ebert 2004). While both plasticity and local adaptation can be useful evolutionary strategies for dealing with environmental heterogeneity and can increase organism fitness in specific sets of conditions, the adoption of flexible vs. fixed phenotypes is thought to be differentially favoured depending on selective circumstances (Alpert and Simms 2002, Ghalambor et al. 2007). Local adaptation, for instance, is hypothesized to be most advantageous when immediate environmental conditions are relatively stable, whereas plasticity is thought to be most advantageous when organisms are subject to greater temporal or spatial heterogeneity (Cook and Johnson 1968).

Although many studies have observed intraspecific variation in seaweeds across hydrodynamic gradients (e.g. Armstrong 1989, Gutierrez and Fernández 1992, Bäck 1993, D'Amours and Scheibling 2007), many have failed to investigate whether variation is due to plasticity or genetic differentiation (e.g. Jackelman and Bolton 1990, Duggins et al. 2003, Kitzes and Denny 2005). So, can it really be assumed that plasticity is common in seaweeds? Differentiating between phenotypic plasticity and genetic differentiation can provide researchers with a great deal of information and raise new research questions. Because these two phenomena arise through unique selective conditions (Alpert and Simms 2002, Ghalambor et al. 2007),

determining whether phenotypic variation reflects one or the other can provide insight into trait evolution (e.g. Roberson and Coyer 2004, Fowler-Walker et al. 2006, Demes and Pruitt 2019), which may allow researchers to predict how they will continue to evolve (Roberson and Coyer 2004, Demes and Pruitt 2019). Moreover, differentiating between plastic and genetically fixed phenotypes is essential for taxonomic studies, as mistaking the former for the latter can lead to incorrect species designations (e.g. Garbary et al. 1978, Demes et al. 2009, Belton et al. 2014). A clear understanding of organismal responses to environmental variation can also help researchers predict how organisms will fare in the face of changing climate (e.g. Richter et al. 2012, Sheth and Angert 2014).

5.2.1 Flow sensing as a requirement for adaptive plasticity across hydrodynamic gradients

Reliable environmental cues are thought to be important for the evolution of adaptive phenotypic plasticity (Levins 1963, DeWitt 1998, Ghalambor et al. 2007, Reed et al. 2010) and these cues would be of little value if organisms could not sense them (Smith 1990, Getty 1996, Schlichting and Smith 2002). Therefore, in order for seaweeds to evolve adaptive plasticity across hydrodynamic gradients, there would need to be (1) a chemical or mechanical cue indicative of flow speed and (2) a biological mechanism for perceiving it. Below I propose two different cues for water motion that depend upon flow speed, and then consider seaweed growth modes as mechanisms for sensing those environmental cues. By integrating principles of fluid dynamics with knowledge of biological mechanisms, I attempt to predict the conditions required for seaweeds to demonstrate adaptive plasticity in response to flow.

Any organism living in an environment with little or no water motion may be subject to the effects of the diffusive boundary layer, which reduces diffusion rates of nutrients, gases, and

other chemicals in and out of living tissue (reviewed in Hurd 2000). While this phenomenon has not been observed to play a role in regulating phenotypic plasticity in seaweeds, it has been shown to be involved in a mechanism utilized by several species of fucoid brown algae for sensing water motion. For example, *Fucus distichus* and *Pelvetia compressa* use DIC depletion within boundary layers as a trigger for gamete release, allowing these seaweeds to coordinate their reproductive output during low tides to avoid turbulent water motion that would negatively impact reproductive success (Pennington 1985, Levitan et al. 1992, Pearson et al. 1998). A boundary layer-mediated chemical cue such as this could potentially be used by any seaweed to regulate phenotypic plasticity in response water motion, as long as flow velocities are slow. As ambient flow speed increases, boundary layer thickness decreases, which increases rates of mass transfer (Wheeler 1980, Gerard 1982, Hurd et al. 1996). Once flow reaches a velocity of approximately 20 cm s^{-1}, boundary layers are effectively minimized and mass transfer becomes saturated, nullifying any inhibitory effects (Hurd 2000). Therefore, any chemical metric of water motion mediated by boundary layer thickness would only be useful in ambient flow speeds slower than 20 cm s^{-1}, since beyond this speed, algae would not be able to perceive further changes in water motion.

For seaweeds to differentiate between higher flow speeds, such as those that occur in wave-swept intertidal habitats (Carrington Bell and Denny 1994, Denny and Gaylord 2002, de Bettignies et al. 2013), another flow perception mechanism would be necessary. One reliable index of water motion for flow velocities above 20 cm s^{-1} is mechanical loading imposed by drag. This phenomenon was first observed in the kelp *Saccharina latissima* by Gerard (1987), who found that longitudinal tension continuously applied to kelp blades caused them to grow narrower and longer. These morphological changes were consistent with those observed in

several kelp species following field transplants between areas of differing wave exposure (e.g. Sundene 1964, Norton 1969, Pace 1972, Gerard and Mann 1979), and so it was concluded that drag was likely the cue being perceived by kelps to facilitate flow-induced plasticity. Associations between mechanical forces and plasticity in kelps have since been demonstrated in *Egregia menziesii* (Kraemer and Chapman 1991b, 1991a) and in *Nereocystis luetkeana* (Koehl et al. 2008, Coleman and Martone 2020, Ch. 2).

In order for drag to be a mechanical indicator of flow speed, there must be a substantial amount of tissue located distal to the growing tissue to act as a drag element (Fig. 5.1). Such a developmental pattern would be consistent with intercalary or diffuse growth in seaweeds. Indeed, I observed in Ch. 2 that *Nereocystis luetkeana* would only exhibit morphological plasticity in blades when tension was applied directly to the intercalary meristem at the blade bases (Coleman and Martone 2020). Accordingly, seaweeds with apical growth, such as *Fucus* or most red algae (Graham et al. 2017), might be unable to sense drag unless it were perceived in a non-growing region of the thallus and somehow communicated to the meristem via a long-distance signaling mechanism, such as a hormone. While there is some evidence that hormones contribute to growth and development in macroalgae (Hart 1982, de Nys et al. 1990, Basu et al. 2002, Kai et al. 2006, reviewed in Tarakhovskaya et al. 2007, see Ch. 3), hormone activity in algae remains largely mysterious and no research to date has connected mechanical signals to hormone activity in any seaweed. How such a response would work in red algae is especially challenging to imagine, as cell-to-cell communication is largely impeded by the lack of plasmodesmata and the presence of pit plugs (Pueschel 1977). In summary, based on our understanding of fluid dynamics and growth modes in seaweeds, adaptive phenotypic plasticity in response to flow speeds less than 20 cm s^{-1} is theoretically possible in any seaweed, but

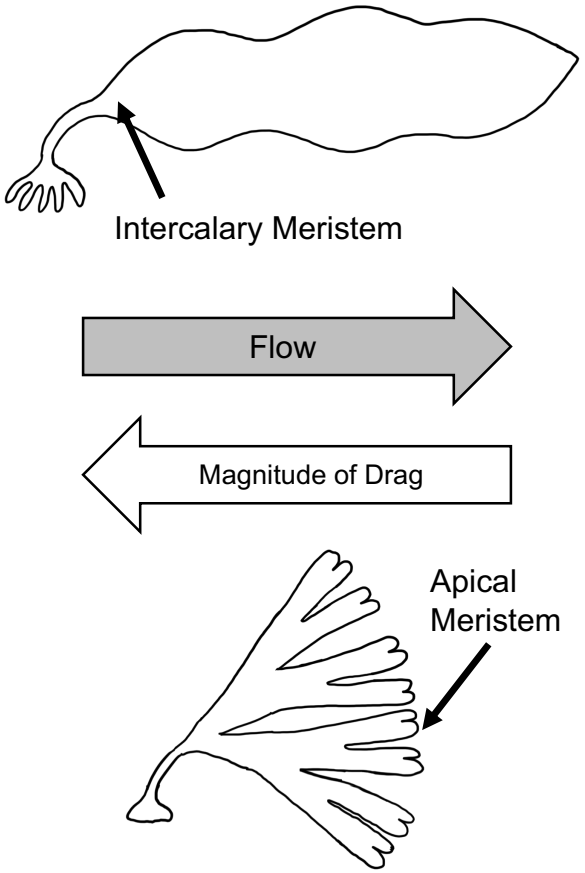

Fig. 5.1. Spatial distribution of drag experienced in flow by seaweeds with different meristem types. Top: a kelp, *Saccharina*; bottom: a fucoid, *Fucus*. Drag imposed on a given point along a seaweed in flow is proportional to the tissue area downstream from that point.

plasticity in response to flow speeds greater than 20 cm s^{-1} should only be possible in seaweeds with intercalary or diffuse growth (Fig. 5.2).

5.2.2 Objectives

In this chapter, I review over 100 years of phycological literature to address the general assumption that phenotypic plasticity across hydrodynamic gradients is common in seaweeds, as well as to assess whether range of flow speed and growth mode are useful predictors of such plasticity. I investigate (1) how often intraspecific variation across hydrodynamic gradients has

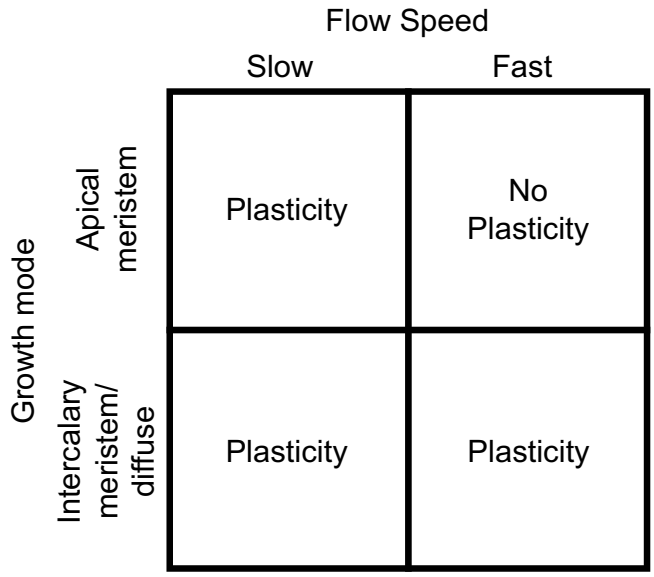

Fig. 5.2. Predictions of when adaptive phenotypic plasticity across hydrodynamic gradients should be possible with respect to ambient flow velocity and growth mode.

been observed in seaweeds, (2) how often the origin of observed variation (i.e., phenotypic plasticity vs. genetic differentiation) is investigated, (3) how often phenotypic plasticity across hydrodynamic gradients has been confirmed in seaweeds, and (4) whether range of flow velocity and growth mode affect the frequency of occurrence of plasticity. I hypothesize that plasticity should be able to occur in any seaweed when flow is slow, but that it should only occur in seaweeds with either intercalary meristems or diffuse growth when flow is fast. I also hypothesize that, due to intercalary meristems being ubiquitous in kelps but uncommon in other algae (Graham et al. 2017), plasticity in high flow will be more common in the brown algae than in the red or green algae.

5.3 Methods

I searched the Web of Science for literature published between 1900 and 2020, using the search terms (alga* OR seaweed*) AND (phenotypic* plastic* OR morpho* OR variab*) AND

(wave* OR current* OR expos* OR hydrodynamic* OR water motion OR water movement OR flow) to capture the broadest range of relevant articles possible. I then read titles and abstracts of literature returned by the search (6885 items) and selected those that discussed phenotypic variation in a seaweed across a gradient of water motion. Articles that only discussed variations in growth rate were ignored, as these would likely only be documenting nutrient limitation by boundary layers (Hurd 2000), which is different phenomenon than the adaptive flow sensing I am seeking evidence of in this chapter. All papers in this initial shortlist (84 items) were read thoroughly to ascertain if they reported primary data documenting phenotypic variation in a seaweed across an environmental gradient associated with water motion (e.g. wave exposure, flow velocity, tensile force, etc.). Articles that did not meet this criterion were discarded. For the remaining articles, I recorded (1) species name, (2) the higher taxonomic group to which the species belongs (red, green, or brown algae), (3) growth modes in question (e.g. apical meristem, intercalary meristem, or diffuse), (4) phenotypes observed, (5) the specific environmental factor attributed to observed phenotypic variation (e.g. wave exposure, flow velocity, or force attributed to drag), (6) whether the maximum value of the relevant environmental factor corresponded to a flow velocity greater than or less than 20 cm s^{-1}, and (7) whether plasticity was demonstrated for each individual study species. I considered plasticity to be demonstrated if the authors of an article reported that changing the relevant environmental factor had a statistically significant effect on the observed phenotype. Plasticity could have been demonstrated in a laboratory or field setting, over any time period, using any method. I excluded instances where authors attributed phenotypic changes to mechanical damage for purposes of this review because damage is out of the organism's control and does not require active flow sensing to take place; this removed three articles from the list. I also read the bibliographies of each article and collected

any relevant papers that I did not find in my initial Web of Science search; data on the same six variables listed above were collected from any additional articles found this way. Species names given in all articles were compared against taxonomic data from AlgaeBase (Guiry and Guiry 2021) and only the most current name was recorded. If two articles described the same data, only one was included in the data set. The final list of articles deemed to contain relevant data consisted of 121 items.

Fisher's exact tests were used to test whether there was an association between taxonomic group and the tendency for plasticity to be (1) tested for and (2) demonstrated. A Fisher's exact test was also used to assess whether, among cases of confirmed plasticity, there was an association between flow speed and meristem type. These tests were performed in R using the fisher.test() function from the R Stats Package (R Core Team 2021).

5.4 Results

I assembled 121 papers documenting a total of 128 instances (paper/species combinations) of intraspecific phenotypic variation across hydrodynamic gradients in 57 species of seaweed (Table 5.1; Fig. 5.3). 105 instances of variation (82.0%) occurred in the brown algae, 16 (12.5%) occurred in the red algae, and 7 (5.5%) occurred in the green algae. Of the instances of variation identified, the origin of that variation was investigated for 45 of them (35% of cases). The origin of variation was found to be investigated a similar proportion of the time in the brown, red, and green algae (Fisher's exact test, $p=0.50$). Among those instances where the origin of variation was investigated, 32 cases of verifiable phenotypic plasticity were identified. Twenty-six of these cases (81.3%) occurred in the brown algae, three occurred in the red algae (9.4%), and three (9.4%) occurred in the green algae. Intraspecific variation was found to be due

Table 5.1. Counts of instances (paper/species combinations) of phenotypic variation and plasticity in response to water flow. Percentages of column totals are given in parentheses.

Group	Unique species	Instances of variation	Instances where plasticity was tested	Instances of verified plasticity
Brown algae	41 (71.9%)	105 (82.0%)	36 (80.0%)	26 (81.3%)
Red algae	10 (17.5%)	16 (12.5%)	5 (11.1%)	3 (9.4%)
Green algae	6 (10.5%)	7 (5.5%)	4 (8.9%)	3 (9.4%)
Total	**57**	**128**	**45**	**32**

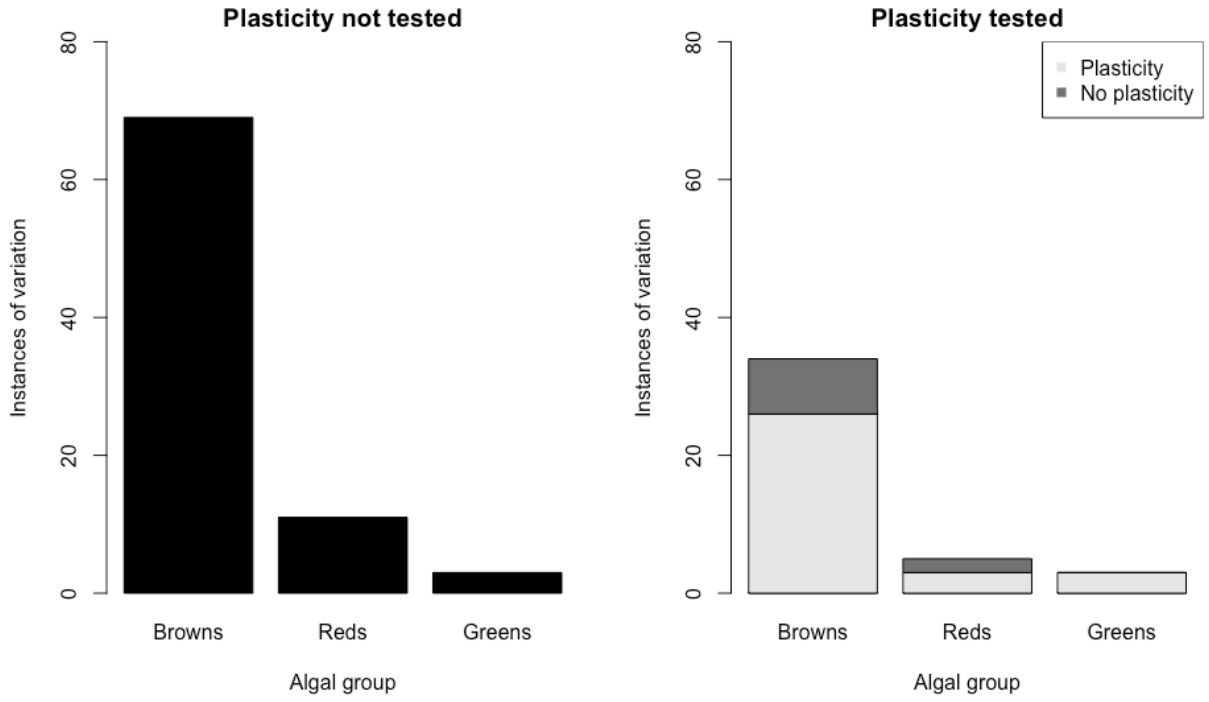

Fig. 5.3. Counts of instances of phenotypic variation in seaweeds across hydrodynamic gradients where (A) plasticity was not tested and (B) plasticity was tested expressed as functions of taxonomic group (brown, red, or green) and whether plasticity was found.

to plasticity a similar proportion of the time in each of the three major taxonomic groups (Fisher's exact test, p=0.45). Most cases of plasticity (66%) occurred in seaweeds with intercalary meristems at fast flow speeds (Fig. 5.4). However, there were also five instances of seaweeds with apical meristems showing plasticity in fast flow (15.6%). Among confirmed cases of plasticity, plasticity in fast flow was more likely to occur in a seaweed with an intercalary

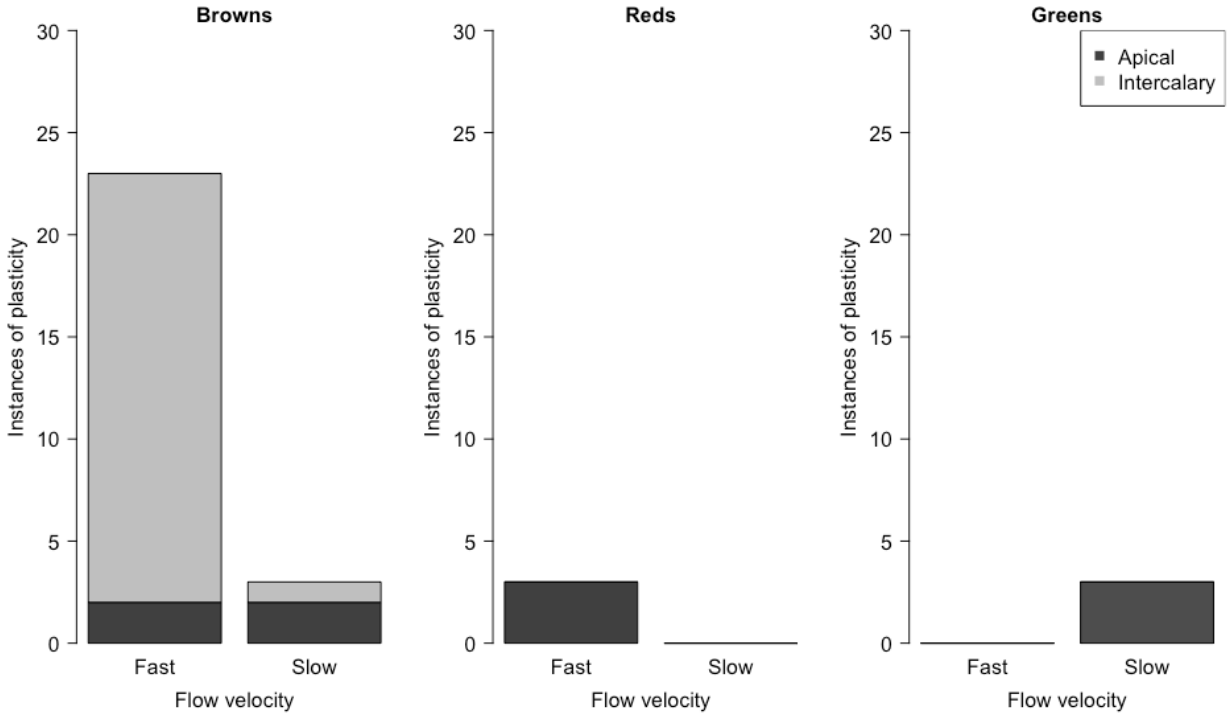

Fig. 5.4. Counts of instances of phenotypic plasticity in seaweeds across hydrodynamic gradients as functions of flow velocity (slow vs. fast) and growth mode (apical vs. intercalary meristem) for (A) brown, (B) green, and (C) red algae.

meristem (Fisher's exact test, p=0.006). There were no observations of plasticity across hydrodynamic gradients occurring in seaweeds with diffuse growth.

5.5 Discussion

Despite broad claims that phenotypic plasticity is common in seaweeds, there was mixed evidence for its prevalence in the phycological literature. Intraspecific variation across hydrodynamic gradients has been well documented in brown algae, but less so in red and green algae. However, only 35% of cases of observed phenotype variation explicitly tested for plasticity. Furthermore, among the cases where the origin of variation was investigated, ten (22%) could not be attributed to plasticity and may represent instances of local adaptation to the hydrodynamic environment. These findings may suggest that assumptions about the prevalence

of phenotypic plasticity across hydrodynamic gradients in seaweeds are not well founded in data. They also serve as reminder to phycologists to consider genetic differentiation as a potential driving force behind intraspecific phenotypic variation.

As with general phenotypic variation, verifiable phenotypic plasticity across hydrodynamic gradients has been well documented in brown algae, but not in red and green algae, with merely three published instances of plasticity each in the red algae and green algae. Furthermore, one instance of red algal plasticity (Steneck and Adey 1976) and all three instances of green algal plasticity (de Senerpont Domis et al. 2003) could not be clearly attributed to changes in water motion by the authors and may instead reflect responses to light, suggesting that there may be even fewer cases of plasticity in response to water motion than indicated. This is a striking finding and it is tantalizing to consider that plasticity in response to water motion could be a trait that is common in brown algae but rare in red and green algae. However, unfortunately, it is not possible to say for sure that the greater abundance of brown algal examples of phenotypic plasticity is actually due to brown algae being more plastic; it may simply reflect a greater number of studies examining trait variation having been conducted in brown algae than in red and green algae. In fact, the observation that a similar proportion of instances of variation represented plasticity in each of the three major taxonomic groups of seaweeds suggests that this is likely the case. To clarify whether brown algae are actually more likely to exhibit plasticity across flow gradients than other seaweeds, more studies will be needed to rigorously examine the abilities of red and green algae to adjust phenotypes in response to flow.

The literature yielded mixed support for my hypotheses that the occurrence of adaptive plasticity would be influenced by flow velocity and growth mode. The largest proportion of cases of plasticity was represented by brown algae with intercalary meristems (almost entirely kelps) in fast flow, which may represent support for my hypotheses. Furthermore, these instances of plasticity were largely consistent with morphological plasticity that was adaptive and mediated by developmental changes (Gerard 1987, Koehl et al. 2008, Charrier et al. 2012, Coleman and Martone 2020, Ch. 2). Additionally, when plasticity takes place in fast flow speeds, it is more likely to occur in a seaweed with an intercalary meristem. Collectively, these observations may suggest that intercalary growth and fast flow are favourable conditions for the evolution of adaptive phenotypic plasticity in response to water motion. However, while this is an intriguing hypothesis, I once again cannot rule out the alternate explanation that there have simply been more studies examining variation and plasticity across flow gradients in kelps than there have been in other seaweeds and that this is inflating the observed frequencies of seaweeds with intercalary meristems exhibiting plasticity in high flow. The fact that plasticity was identified a similar proportion of the time in each of the three taxonomic groups would support this alternate explanation.

The data contradict my hypotheses with five instances of seaweeds with apical meristems showing plasticity in high flow. Taken at face value, this would indicate that there must be an additional mechanism beyond those proposed above making these observations possible. Seaweeds in these instances could, for example, sense drag in proximal tissue and communicate that signal to apical meristems to facilitate growth changes. However, closer examination of these individual cases may suggest that the situation is more nuanced than it initially appears. In one case, the brown alga *Turbinaria ornata* developed pneumatocysts when transplanted from

high to low wave exposure, compensating for dispersal limitation (Stewart 2006). This phenomenon could be the result of waves mechanically removing pneumatocysts in high flow, especially given that wave-exposed populations had smaller thalli than wave-protected thalli (Stewart 2006). In another instance. Steneck and Adey (1976) showed that the crustose coralline red alga *Lithophyllum kaiseri* would change its morphology when transplanted across a wave exposure gradient, becoming more textured and "branched" in high wave exposure. While this alga has functionally apical growth (Johansen 1981), it is unlikely that the plasticity it showed in this study was mediated by drag due to its crustose morphology and inflexible tissues. It should also be noted that the authors could not disentangle the effects of wave exposure from those of light and grazing in this experiment, and so the plasticity observed may not be due to flow at. Overall, I suggest that some of the documented instances of plasticity in seaweeds with apical meristems in fast flow may not involve a flow perception mechanism and therefore would fall outside of the scope of my hypotheses. However, further study of the biological systems in question would be required to say this confidently.

In two additional instances of seaweeds with apical meristems showing phenotypic plasticity in high flow, the red algae *Mazzaella splendens* and *Mazzaella linearis* changed morphology when transplanted across wave exposures (Shaughnessy 2004). This may be the most compelling evidence uncovered in this review that some seaweeds with apical meristems are capable of responding to changes in flow. However, curiously, the changes observed in these red algae were maladaptive in the context of the environments they were transplanted into (i.e., growing broader in wave-exposed areas; Shaughnessy 2004). This raises questions about the developmental or genetic mechanisms are taking place in these *Mazzaella* species to facilitate plasticity and how they differ from mechanisms in kelps. These cases merit further examination.

There were several additional observations made in this analysis that are interesting in the context of my original hypotheses. Firstly, while many species of kelps clearly demonstrate plasticity in blade morphology when their flow environment is manipulated (e.g. Druehl and Kemp 1982, Buck and Buchholz 2005, Fowler-Walker et al. 2006, Koehl et al. 2008), a small handful do not. One of these is the feather boa kelp, *Egregia menziesii*. While this alga does develop smaller bladelets and thicker rachi in increased wave exposure (Abbott and Hollenberg 1976, Blanchette et al. 2002, Henkel et al. 2007), transplantation experiments have failed to attribute this variation to plasticity (Blanchette et al. 2002). Interestingly, *Egregia* is morphologically unique among kelps and has an unusual intercalary meristem that is located distally when thalli are mature (Burnett and Koehl 2020). This distal position may interfere with the ability of this kelp's intercalary meristem to perceive flow, similarly to an apical meristem, which could explain the lack of observed plasticity.

A large number of studies have documented phenotypic variation across hydrodynamic gradients in brown algae that are not kelps (i.e., not representatives of the Order Laminariales) (e.g. Norton 1969, South and Hay 1979, De Paula and de Oliveira 1982, Blanchette 1997, Mueller et al. 2015). However, unlike in the kelps, very few studies have been able to demonstrate plasticity in these non-kelp brown algae, which mostly exhibit apical growth. A genus that has received a great deal of attention is the genus *Fucus*, which has been the subject of numerous studies documenting phenotypic variation across hydrodynamic gradients worldwide (eg. Knight and Parke 1950, Jordan and Vadas 1972, Rice et al. 1985, Kalvas and Kautsky 1993, Coleman and Muhlin 2008). In spite of the large body of research focusing on this genus, which includes multiple transplant experiments (Sideman and Mathieson 1985, Blanchette 1997), no study has been able to attribute the morphological variation often observed in *Fucus* to plasticity

(although one did show that tissue material properties were a plastic trait; Molis et al. 2015). I suggest that the variability in morphology often observed in this genus is due to widespread, fine-scale genetic differentiation, a hypothesis supported by research that shows that *Fucus* can show detectable genetic differentiation at scales of metres (Coyer et al. 2003, Tatarenkov et al. 2007). Interestingly, the only non-kelp brown alga that shows clear morphological plasticity that resembles that shown by kelps in fast flow conditions is *Saccorhiza polyschides* (Norton 1969), which possesses an intercalary meristem (Norton 1970).

Several studies observed morphological variation across exposure gradients in the red alga *Chondrus crispus* (e.g. Lilly 1968, MacFarlane 1968, Chen and Taylor 1980, Menéndez and Fernández 1989, Gutierrez and Fernández 1992). However, in spite of multiple attempts, no study has been able to show that manipulating flow leads to morphological changes in this alga (Floc'h 1969, Chen and Taylor 1980b), suggesting that morphological variation may be due to genetic differentiation.

5.6 Conclusions

Phenotypic plasticity is often assumed to be common in seaweeds but is rarely explicitly tested. In this chapter, I considered biomechanical and developmental mechanisms that might facilitate adaptive phenotypic plasticity across hydrodynamic gradients in seaweeds in order to clarify the likelihood of plasticity occurring and to make predictions about when such plasticity might be possible. I hypothesized that plasticity should be possible in any seaweed at slow flow velocities, where flow would be sensed chemically through boundary layers, but that it should only be possible in seaweeds with intercalary or diffuse growth at fast flow velocities, where flow would be sensed mechanically through drag. I tested my hypotheses by conducting a

thorough review of the literature on phenotypic variation and plasticity in seaweeds along hydrodynamic gradients. I found considerable evidence for intraspecific phenotypic variation across flow gradients in brown algae, but limited evidence for variation in red and green algae. Among the instances of variation found, plasticity was only tested in 35% of studies. Substantial evidence of phenotypic plasticity was identified in brown algae, but very little evidence was found in red or green algae, for which there were at most three cases (9% of cases of plasticity) each. The data provided mixed support for my hypotheses regarding the effect of flow speed and growth mode on the occurrence of plasticity. By far the largest proportion of instances of plasticity occurred in seaweeds with intercalary meristems at high flow speeds, which may represent support for my hypotheses. Alternatively, this pattern may simply reflect the fact that there have been more studies of plasticity conducted on brown algae than on red or green algae. The observation that plasticity was documented within each taxonomic group a similar proportion of the time supports this explanation. However, researchers should be warned that generalizations about plasticity in red and green algae are based on very little empirical evidence. The data contradict my hypotheses in five cases where seaweeds with apical meristems also exhibited plasticity in high flow. More studies are needed to clarify the ability of red and green algae to respond to changes in flow environment. Growth mode and flow conditions should be considered before making assumptions about phenotypic plasticity in response to flow in seaweeds, and the possibility that intraspecific trait variation may reflect genetic differentiation/local adaptation should not be discounted.

6. Conclusions

6.1 Major findings of this work

With this thesis, I endeavoured to improve our understanding of the developmental mechanisms underlying morphological plasticity across hydrodynamic gradients in kelps. To this end, I first sought to better characterize the growth response of blades of the kelp *Nereocystis luetkeana* to mechanical loading that would normally be imposed by drag (Ch. 2). The experiments conducted for this purpose revealed that (1) *Nereocystis* blades grow continuously narrower and longer with increased application of tension up to the point that the blade tissue breaks, suggesting that this kelp is capable of fine tuning its morphology to a broad range of hydrodynamic environments, (2) rotating the direction of applied tension rotates the orientation of the growth response, which indicates that the developmental process underlying the plasticity takes place largely at the level of individually stimulated cells and likely does not involve long-distance signaling, (3) the morphology of mechanically stimulated blade tissue is reflected in the morphology of meristoderm cells, indicating that morphological changes induced by mechanical loading stem from changes in the direction of meristoderm cell growth and/or division, and (4) applying a mechanical stimulus to distal non-growing blade tissue does not induce a growth response in meristematic tissue, further suggesting that a long-distance signaling mechanism is unlikely to be involved in regulating kelp plasticity.

Next, I investigated whether auxin signaling could play a role in mediating kelp growth responses to mechanical loading (Ch. 3). I showed that applying exogenous auxin at a concentration of 10^{-5} M affected the morphology of kelp tissues, causing them to grow relatively long and narrow, but did not affect overall growth in terms of biomass. While this is not clear

evidence that auxins mediate morphological plasticity in kelps, the morphological effects of auxin and mechanical loading are strikingly similar.

I examined whether plasma membrane-bound stretch activated Ca^{2+} channels might be involved in the perception of mechanical cues that induce morphological plasticity (Ch. 4). I found that reducing the ambient Ca^{2+} concentration by 50% had no effect on the ability of *Macrocystis pyrifera* to sense and respond to mechanical loading. This could indicate that (1) stretch activated Ca^{2+} channels and Ca^{2+} signaling are not involved in kelp mechanoperception, (2) stretch activated Ca^{2+} channels are involved, but a 50% reduction in Ca^{2+} concentration is not great enough to interfere with mechanoperception, or (3) Ca^{2+} signaling is involved in kelp mechanoperception, but the Ca^{2+} ions are derived from internal stores rather than plasma-membrane bound channels. In this chapter, I also show that a reduction in ambient Ca^{2+} concentration of more than 50% is lethal for *Macrocystis*.

Finally, I investigated how often phenotypic plasticity across hydrodynamic gradients has been clearly documented in different seaweeds and considered whether mechanisms of flow perception could constrain the evolution of this trait (Ch. 5). I found that researchers have only explicitly tested whether observations of intraspecific phenotypic variation across hydrodynamic gradients in seaweeds are due to plasticity 35% of the time. Additionally, when plasticity across flow gradients has been clearly documented, it has been found considerably more often in brown algae, especially those with intercalary meristems, than it has in other groups of seaweeds. This could reflect that intercalary meristems have facilitated the evolution of phenotypic plasticity in kelps by acting as flow sensors, but it is also possible that there have simply been more studies on intraspecific variation in brown algae with intercalary meristems than there have been in other macroalgae. More research is needed to clarify the ability of red and green algae to show

plasticity in response to flow. Researchers should be aware that the assumption that plasticity across flow gradients is very common in seaweeds is based on very little evidence.

6.2. A tentative model for the developmental mechanism underlying morphological plasticity across hydrodynamic gradients in kelps

The ultimate effect of tensile stimuli on kelp blade tissue appears to be a reallocation of tissue growth from the axis perpendicular to the direction of the tension into the axis parallel to the direction of the tension (Coleman and Martone 2020, Ch. 2). This response to mechanical loading is dose-dependent, with blades growing progressively longer and narrower as the magnitude of tensile force increases (Coleman and Martone 2020, Ch. 2). Changes in tissue morphology are reflected in the average morphology of corresponding meristoderm cells, which, especially when combined with the observed sensitivity of the plastic response to the direction of mechanical stimulation, suggests that the effects of mechanical loading on blade growth is rooted in the activity of individually stimulated cells (Ch. 2). This is further supported by the observed lack of response to tensile stimuli applied distantly from the meristem (Ch. 2). Gerard (1987) suggested – but did not explicitly show – that longitudinally oriented tension induced the cells of *Saccharina latissima* to preferentially divide longitudinally instead of transversely with respect to the main axis of the kelp blade. My data are consistent with this hypothesis, but it remains unclear how much cell division and cell elongation each contribute to the increased growth of cells and tissues observed in whichever direction mechanical loading was applied. Gerard's cell division hypothesis could be verified by microscopically visualizing whether the application of tension affects the frequency and direction of meristoderm cell divisions.

Assuming morphological plasticity in response to mechanical loading is the result of changes in the direction of elongation and/or division of individually stimulated meristoderm cells, the process of kelps modifying their blade morphology in response to a tensile stimulus most likely begins with mechanical deformation of meristoderm cell walls. Researchers examining responses to mechanical stimuli in plants and fungi have largely proposed that perception of any mechanical perturbation will begin with wall deformation (e.g. Jaffe et al. 2002, Braam 2005, Chehab et al. 2008, Monshausen and Gilroy 2009), and so I suggest it is plausible that the same is true in kelps, especially given the many similarities in cellular structure between kelps and plants (Kloareg and Quatrano 1988, Michel et al. 2010, Starko et al. 2018).

The steps in the developmental process underlying kelp morphological plasticity that immediately follow cell wall deformation remain unclear. In Chapter 4, I proposed that mechanical deformation of meristoderm cell walls might trigger the opening of stretch activated Ca^{2+} channels embedded in plasma membranes, resulting in an influx of Ca^{2+} that would initiate a signaling cascade that would ultimately yield changes in cellular growth patterns. This has been repeatedly proposed as a potential mechanism of mechanoperception in land plants (e.g. Jaffe et al. 2002, Chehab et al. 2008, Monshausen and Gilroy 2009, Landrein and Ingram 2019). However, experiments in which I subjected mechanically perturbed kelps to reduced ambient Ca^{2+} concentrations failed to produce any evidence that limiting Ca^{2+} availability could inhibit the kelp's growth response to tension (Ch. 4). Similar experiments have been able to demonstrate that the application of Ca^{2+} chelators can weaken plant responses to mechanical stimuli (Jones and Mitchell 1989) and that removal of Ca^{2+} from the environment can inhibit electrochemical responses to touch in characean green algae (Kaneko et al. 2005). While reducing the ambient Ca^{2+} concentration failing to alter plasticity in *Macrocystis* could indicate that Ca^{2+} signaling is

not involved in mechanoperception, it is also possible that a 50% reduction in Ca^{2+} concentration – the greatest reduction I could apply without swiftly inducing mortality (Ch. 4) – may not have been sufficient to effectively inhibit an influx of Ca^{2+} via stretch activated channels. I suggest that further research will be necessary to convincingly say one way or another if stretch activated Ca^{2+} channels (or Ca^{2+} signaling in general) are involved in kelp mechanoperception. Given the apparently great sensitivity of kelps to reductions in Ca^{2+} availability, such research could examine whether mechanical stimulation causes increases in cytosolic Ca^{2+} or upregulation of Ca^{2+}-sensitive genes in kelps, as it does in land plants (e.g. Braam and Davis 1990, Knight et al. 1992).

An intermediate step in the developmental process underlying kelp plasticity could involve auxin signaling. I observed in Chapter 3 that a 10^{-5} M treatment of exogenous auxin caused *Nereocystis* blade tissue to grow relatively long and narrow, which is very reminiscent of the observed effects of longitudinal mechanical loading on the same tissues (Ch. 2). Furthermore, auxin is known to occur naturally in *Nereocystis* (Van Overbeek 1940). While the experiment described in Chapter 3 does not directly tie auxin activity to kelp morphological plasticity, it is tantalizing to imagine that mechanical stimulation might induce an increase in auxin concentration in kelp meristoderm cells. For example, auxin activation could be a downstream effect of a Ca^{2+} influx facilitated by stretch activated Ca^{2+} channels, assuming that such structures are involved in kelp plasticity (Ch. 4). Elevated auxin concentrations likely cause kelp meristoderm cells to grow longer and narrower by increasing the extensibility of the cell walls, as this is how auxins promote cell elongation in plants (Braybrook and Peaucelle 2013, Majda and Robert 2018). The anisotropic growth observed in kelp tissues treated with 10^{-5} M auxin could reflect pre-existing anisotropy in the material properties of meristoderm cell walls, most

likely driven by cellulose microfibril patterning (Kloareg and Quatrano 1988), favouring expansion in the longitudinal axis (Baskin 2005, Majda and Robert 2018). These hypotheses would need to be clarified by explicitly testing the effects of auxin application on the material properties of different regions of kelp meristoderm cell walls, possibly using atomic force microscopy (Tesson and Charrier 2014). The involvement of auxin in morphological plasticity of kelps also needs to be clarified, as we currently have no direct evidence that it is. An initial step in this follow-up research could be to measure and compare endogenous auxin concentrations in kelp tissues that have and have not been subjected to tension.

Regardless of whether auxin is involved in regulating morphological plasticity in kelps, mechanical stimulation likely influences the direction of growth and/or division of meristoderm cells through effects on the cytoskeleton and cell wall. In plant cells, the direction of growth is largely dictated by the orientation of cellulose microfibrils embedded in the walls, which is in itself determined through the activity of cortical microtubules (Baskin 2005, Wolf et al. 2012). The orientation of cellulose microfibrils controls the extensibility of the plant cell wall, and the direction of cell growth is determined by the differential extensibility of wall faces (Baskin 2005). In brown algae, actin filaments determine the orientation of cellulose microfibrils in cell walls (Karyophyllis et al. 2000, Katsaros et al. 2002, 2006), which, much like in plants, can control the direction of cell growth (Karyophyllis et al. 2000, Katsaros et al. 2006). Additionally, the cell wall and actin cytoskeleton play essential roles in the formation and fixation of cell polarity (Fowler and Quatrano 1995, Katsaros et al. 2006). Mechanical loading might induce meristoderm cells to grow and/or divide in the longitudinal axis (with respect to the orientation of the blade), at least in part, by modifying cell wall material properties. Stretching of the meristoderm cells caused by tension on the kelp blade could, for example, induce anisotropic

stiffening of cell walls that could cause subsequent cellular growth to proceed in the direction of tension. It has been shown that tension causes kelp cells to incorporate more carbon into their cell walls (Kraemer and Chapman 1991a) and affects the crystallinity of cellulose embedded in kelp cell walls (Hackney et al. 1994). These phenomena could reflect the kelp modifying wall composition to increase stiffness. Such a mechanism could potentially work in concert with an influx of auxin to stiffen some wall faces while simultaneously loosening others to direct growth. Wall property modification could also be initiated by an influx of Ca^{2+} facilitated by stretch activated channels. The hypotheses that wall properties direct the division of meristoderm cells and that these wall properties are modified by mechanical loading to change blade morphology could be investigated using atomic force microscopy (Tesson and Charrier 2014). Wall stiffness could be measured in the longitudinal and lateral faces of meristoderm cells and compared between tissues that had and had not been stretched.

6.3 Concluding remarks

The research described in this thesis ultimately represents an early investigation of the developmental mechanisms underlying morphological plasticity across hydrodynamic gradients in kelps. Although we have long known that kelps can exhibit morphological plasticity (e.g. Sundene 1964, Gerard and Mann 1979, Gerard 1987), until now little research had been conducted to elucidate the process by which it occurs. While the current work improves our understanding of kelp morphological plasticity, fundamental questions remain unanswered. Are changes in kelp blade morphology brought about by changes in the frequency of cell divisions in different axes? What cell signaling mechanisms are involved? Is the direction of kelp meristoderm cell division controlled by wall material properties? Are the developmental

mechanisms for sensing and responding to flow unique to brown algae? Much more research will be needed to answer these and other questions.

The morphological plasticity shown by kelps such as *Nereocystis* and *Macrocystis* across hydrodynamic gradients is a remarkable phenomenon that likely contributes greatly to the ability of these species to occupy a range of hydrodynamic environments (Ch. 2). A mechanistic understanding of how kelps perceive and respond to flow may help us clarify the functioning of kelp forests and anticipate changes in associated coastal ecosystems. Research has already indicated that warming oceans may interfere with the ability of *Nereocystis* to adjust its blade morphology, with likely downstream effects on productivity (Supratya et al. 2020). Thus, greater knowledge of the developmental processes underlying plasticity could have value for conserving kelps and kelp forest ecosystems.

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Appendices

Appendix A – Supplementary Information for Chapter 2

Table A1: ANOVA table for model of change in stress from stress magnitude experiment

Response	Explanatory	df	Sum Sq	Mean Sq	F	p
Change in stress	Weight	1	64.3	64.3	2.81	0.13
	Kelp	3	130.6	43.5	1.91	0.20
	Weight:Kelp	3	37.3	12.4	0.54	0.66
	Residuals	9	205.6	22.8		

Table A2: ANOVA tables for load magnitude experiment (with stress as predictor)

Response	Explanatory	df	Sum Sq	Mean Sq	F	p
Change in blade length (ΔL_B)	Stress	1	54.1	54.1	17.3	0.001
	Kelp	3	12.7	4.22	1.35	0.30
	Stress:Kelp	3	12.1	4.02	1.29	0.32
	Residuals	13	40.6	3.12		
Change in blade width (ΔW_B)	Stress	1	115.8	115.8	30.8	<0.001
	Kelp	3	29.9	9.95	2.65	0.093
	Stress:Kelp	3	21.6	7.20	1.92	0.18
	Residuals	13	48.8	3.75		
	Stress	1	2.94	2.94	0.60	0.45
	Kelp	3	6.00	2.00	0.41	0.75
	Stress:Kelp	3	6.67	2.22	0.46	0.72

Change in blade thickness (ΔT)	Residuals	13	63.3	4.87		
Change in ruffle (ΔR)	Stress	1	2.37	2.37	10.0	0.007
	Kelp	3	2.72	0.91	3.83	0.036
	Stress:Kelp	3	0.17	0.058	0.25	0.86
	Residuals	13	3.08	0.24		
Change in blade wet mass (ΔM_B)	Stress	1	18.5	18.5	7.90	0.015
	Kelp	3	11.8	3.94	1.68	0.22
	Stress:Kelp	3	3.18	1.06	0.45	0.72
	Residuals	13	30.4	2.33		
Change in blade area (ΔA_B)	Stress	1	20.4	20.4	5.55	0.035
	Kelp	3	24.1	8.02	2.18	0.14
	Stress:Kelp	3	26.7	8.90	2.42	0.11
	Residuals	13	47.8	3.68		

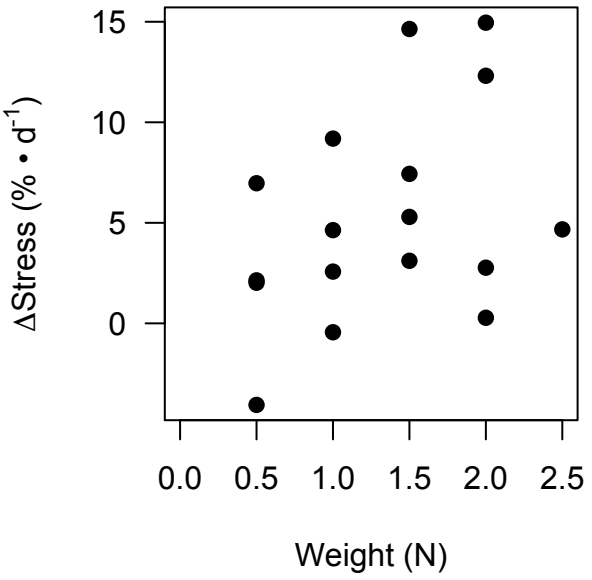

Fig. A1. Change in stress experienced by *Nereocystis* blades at 10 cm from the origin over the course of experimental growth periods expressed as a function of weight applied.

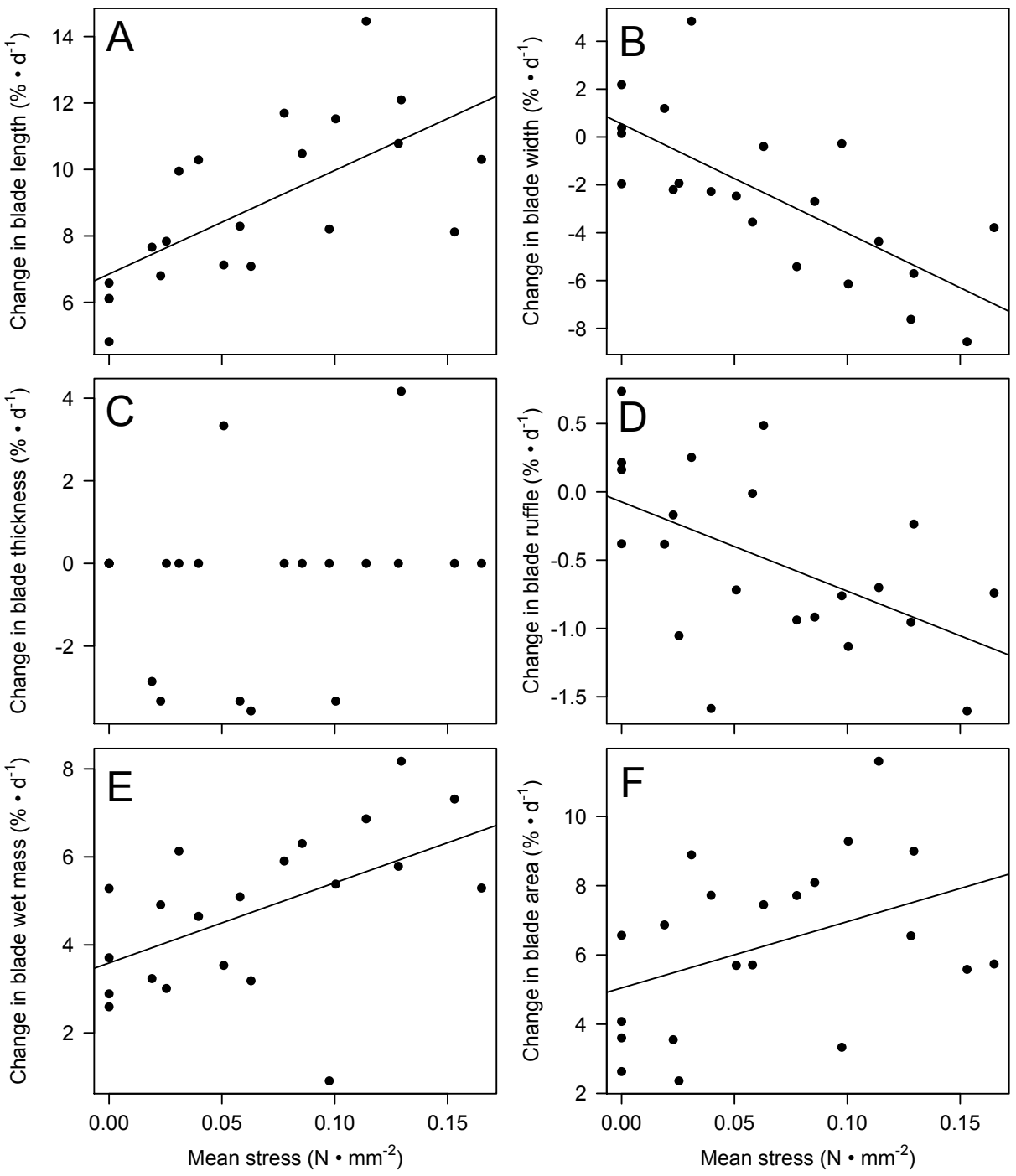

Fig. A2. Change in morphological characters of *Nereocystis* blades expressed as functions of mean stress applied during load magnitude experiment. OLS regression lines indicate a statistically significant relationship between mean stress and the specified response variable. Change in blade length = ΔL_B; change in blade width = ΔW_B; change in blade midline thickness = ΔT; change in blade ruffle = ΔR; change in blade wet mass = ΔM_B; change in blade area = ΔA_B.

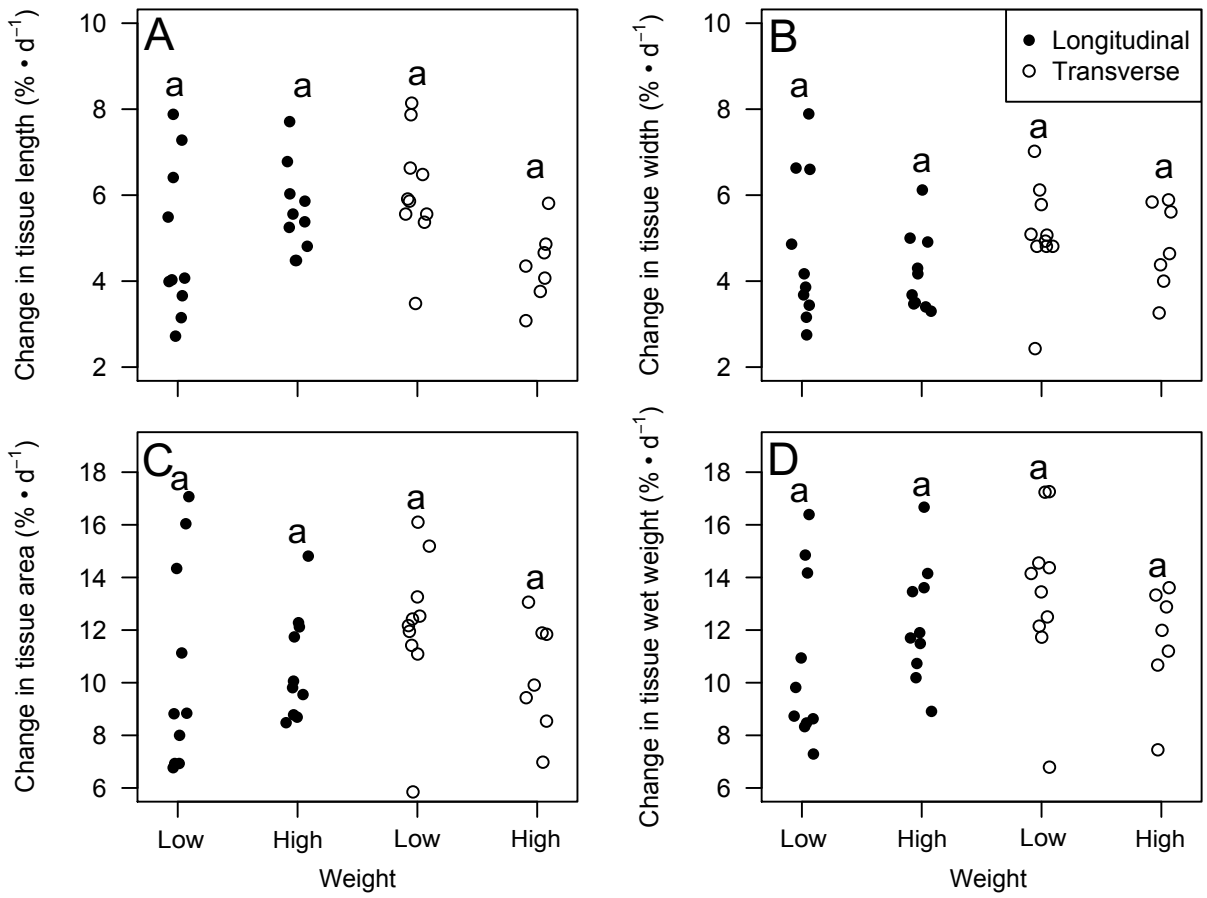

Fig. A3. Change in morphological characters of kelp tissue samples used in load direction experiment expressed as functions of weight and orientation. Change in tissue length = ΔL_S; change in tissue width = ΔW_S; change in tissue area = ΔA_S; change in tissue wet weight = ΔM_S. Lowercase letters denote significantly different groups as indicated by a Tukey-Kramer post-hoc test.

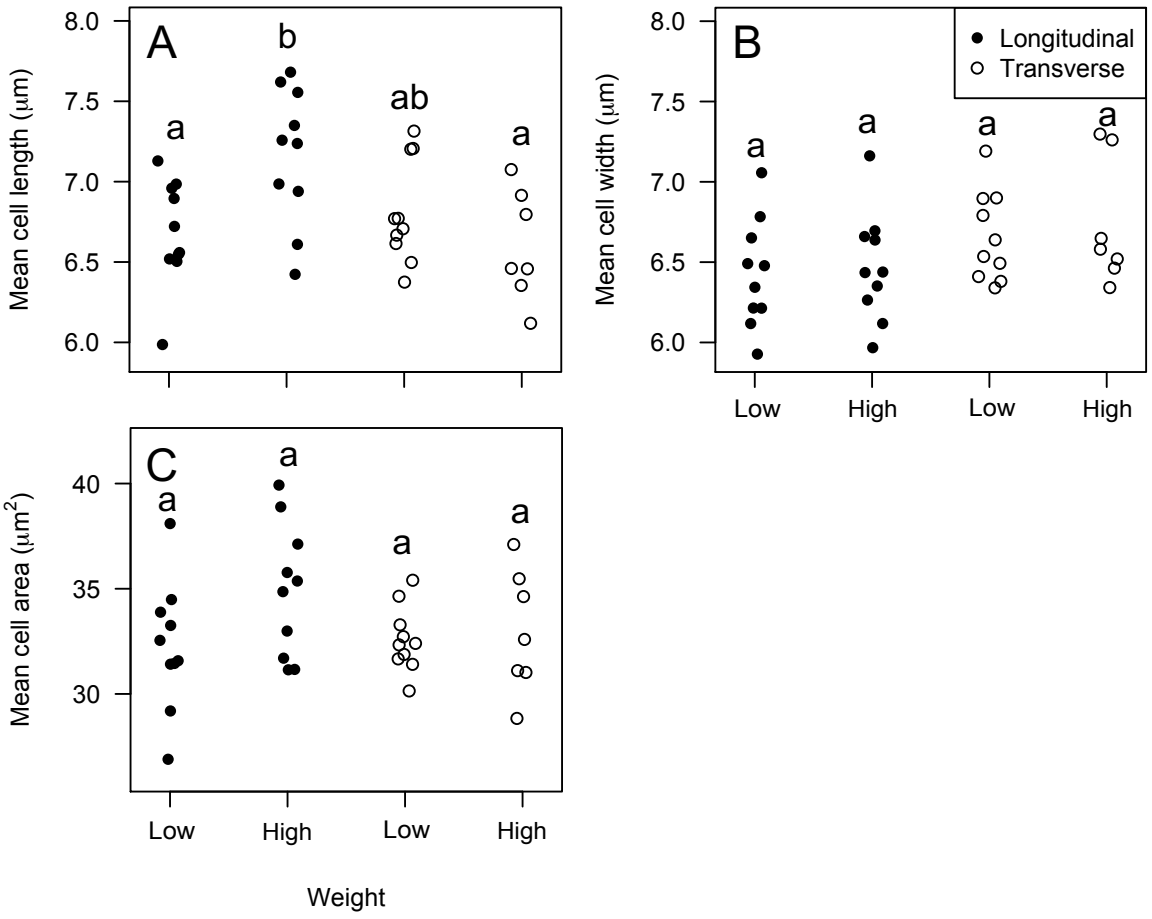

Fig. A4. Mean meristoderm cell morphological characters of kelp tissue samples used in load direction experiment expressed as functions of weight and orientation. Mean cell length = ΔL_C; mean cell width = ΔW_C; mean cell area = ΔA_C. Lowercase letters denote significantly different groups as indicated by a Tukey-Kramer post-hoc test.

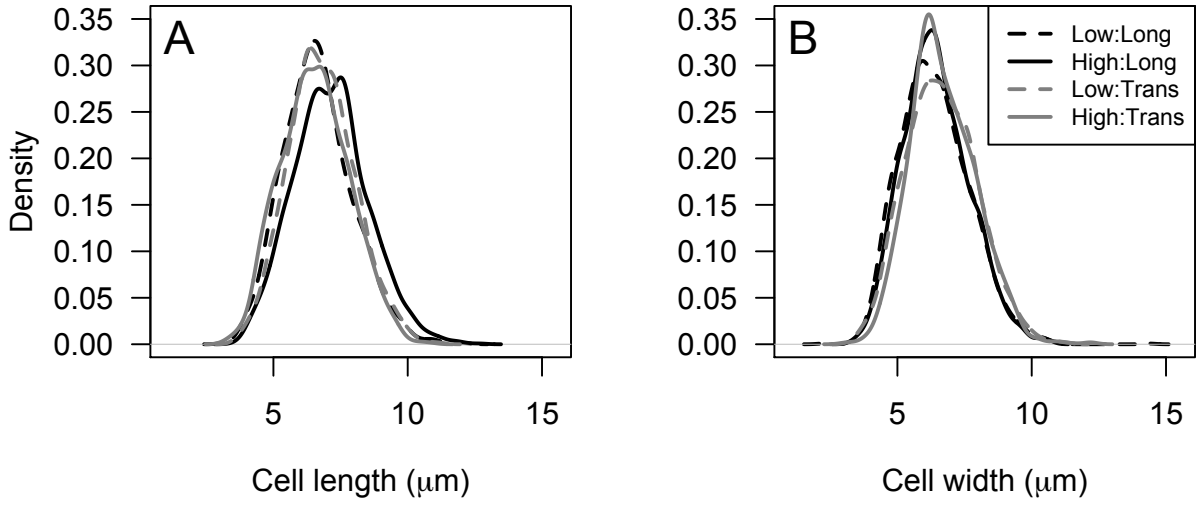

Fig. A5. Density plots of (A) lengths and (B) widths of *Nereocystis* meristoderm cells as functions of weight and orientation applied during load direction experiment.

Appendix B – Supplementary Information for Chapter 3

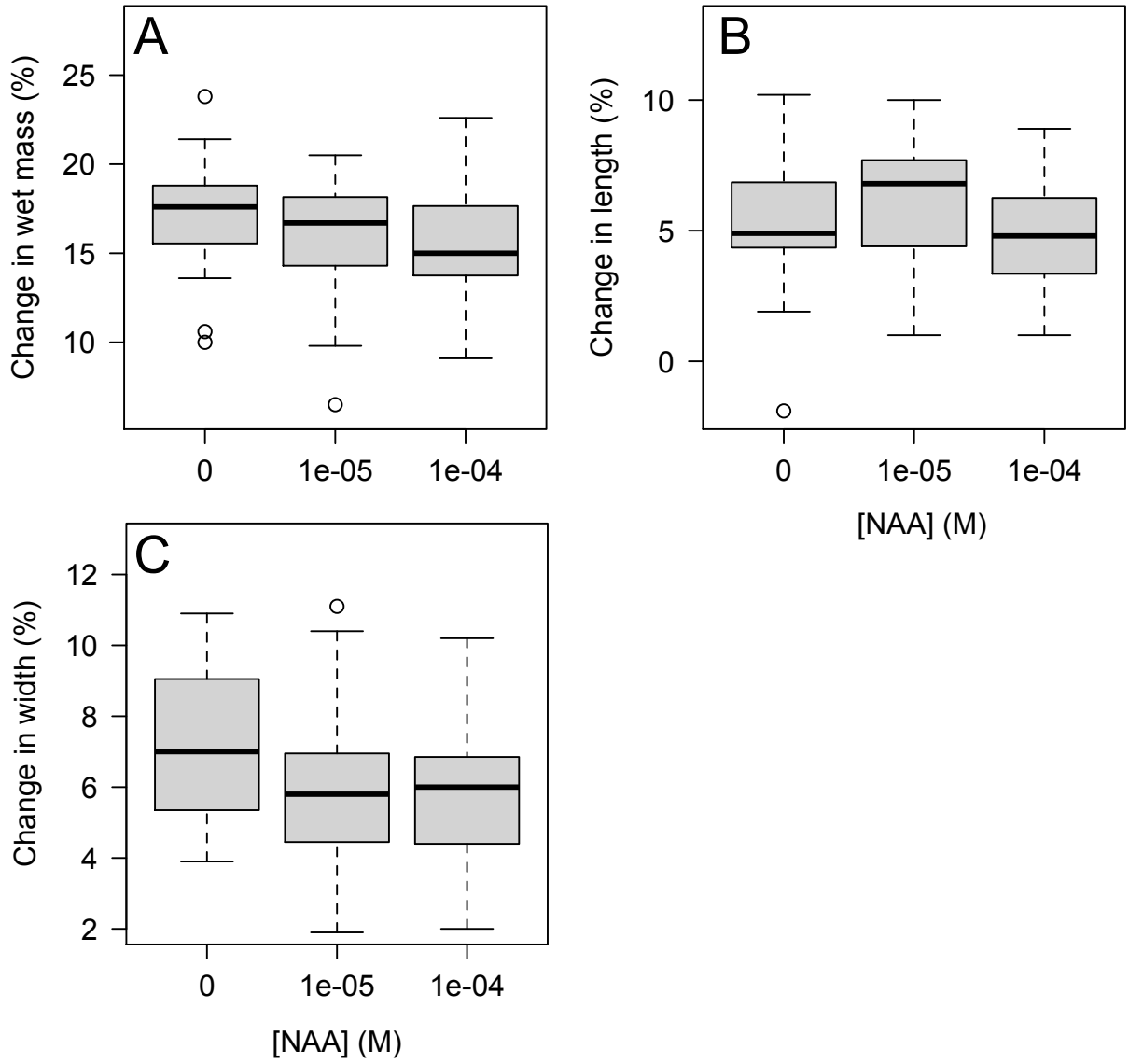

Fig. B1. Change in morphological variables of *Nereocystis* tissue disks expressed as functions of NAA concentration.

Appendix C – Supplementary Information for Chapter 4

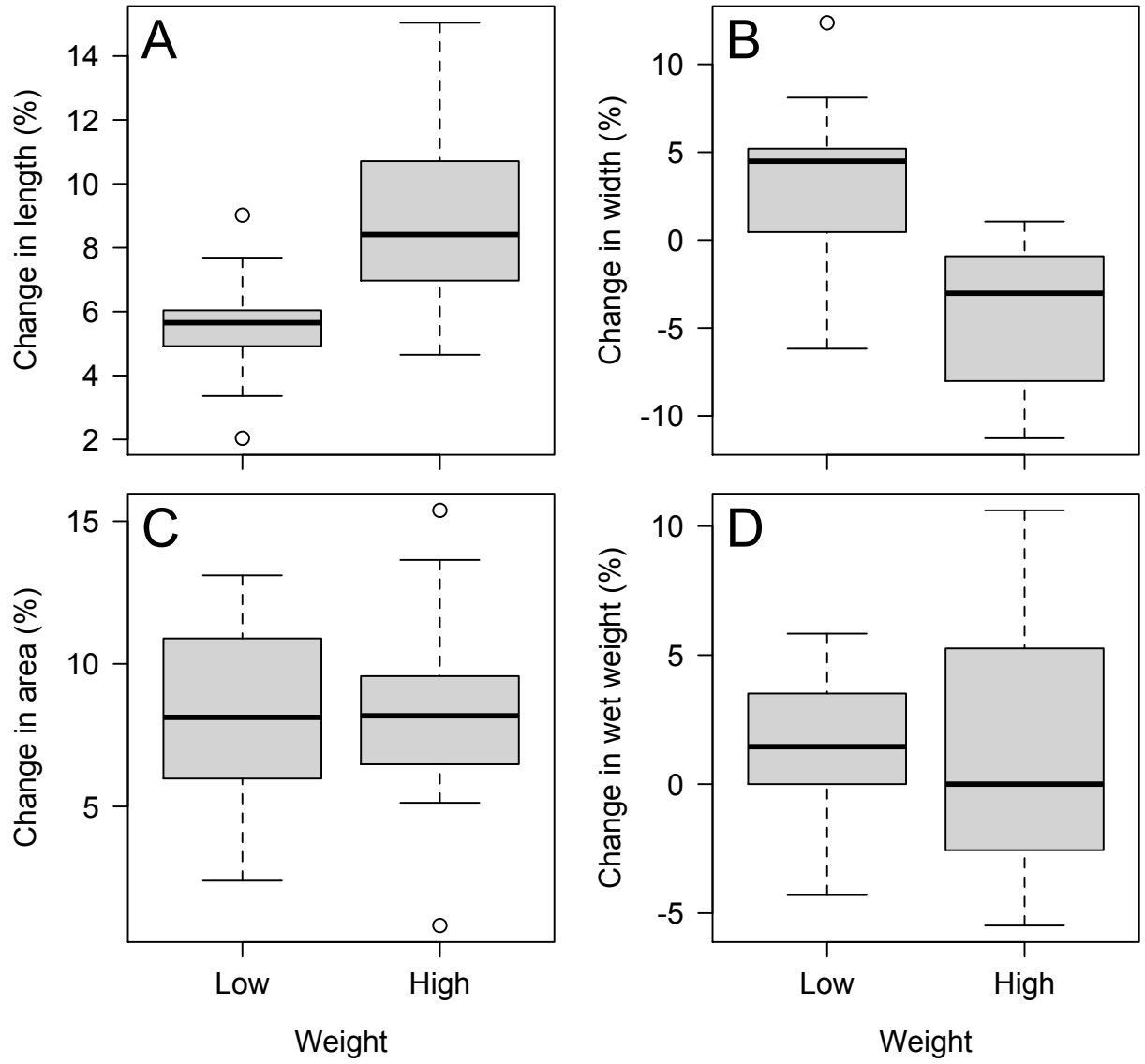

Fig. C1. Change in morphological characteristics of surviving *Macrocystis pyrifera* from calcium exclusion experiment expressed as functions of weight applied.

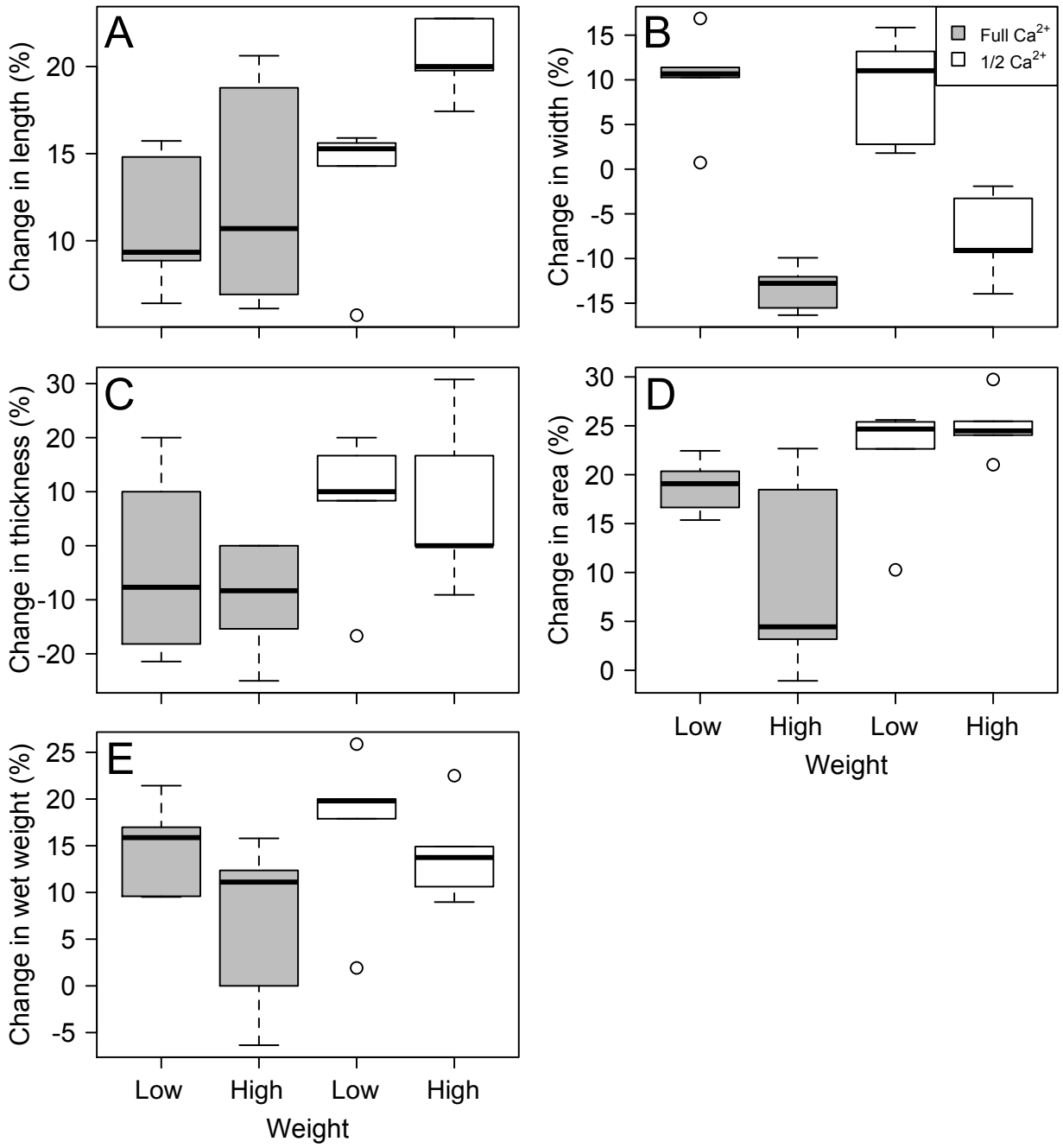

Fig. C2. Change in morphological characteristics of surviving *Macrocystis pyrifera* from calcium gradient experiment expressed as functions of weight applied and calcium concentration.