Co-Optimization of Duckweed Biomass, Nutritional Quality & Input-Use Efficiency for Human Consumption in Space

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1. ABSTRACT

Development of a regenerative, nutritious, and reliable food source is essential for long-duration human spaceflight, such as a mission to Mars. Duckweed (species in the family Lemnaceae) is uniquely suited for growth in space as it has a high growth rate and high nutritional content, which can provide astronauts with a constant food supply rich in protein, healthful fats, vitamins, and other crucial micronutrients. This thesis provides a comprehensive literature review of future human spaceflight plans and associated challenges, health issues unique to astronauts, duckweed biology and nutrient composition, and the mechanisms of action of oxidants (reactive oxygen species) as well as the antioxidant micronutrients zeaxanthin, lutein, vitamin E (α-tocopherol), and pro-vitamin A (β-carotene). Based on this review, this project also sought to identify conditions that minimize the energy resources needed to grow the duckweed species *Lemna gibba* (L.), while simultaneously maximizing its biomass output and nutritional quality. Specifically, the growth light intensity required for maximal production of several essential human micronutrients was ascertained. Production of several antioxidants and other micronutrients was maximal under low to moderate growth light intensities, but concentrations of the essential antioxidant zeaxanthin only peaked under very high growth light intensity. In addition, this thesis describes a protein assay used with *L. gibba*, which confirmed that duckweed's protein content was remarkably high. It was found that the growth light conditions under which production of all duckweed micronutrients can be co-optimized depends on the reference basis used and differs for amount of nutrient produced per plant area and per plant dry biomass. Ultimately, a trade-off will likely have to be made to co-optimize production of those macro- and micronutrients that are most physiologically beneficial to astronauts during spaceflight. Overall, the findings of this thesis, although highlighting the need for more research on defining optimal growth conditions, confirm duckweed as an attractive option for future long-duration human spaceflight missions, as it is suitable for production of protein and essential micronutrients that offer protection from deep space radiation.

2. INTRODUCTION

The extreme environment in space, with microgravity and intense radiation, causes physiological changes in astronauts and results in a unique set of health issues that must be adequately addressed in order to enable future long-duration missions to deep space. National

space policy currently asserts that humans will return to the surface of the Moon by 2024, with plans to set foot on Mars shortly thereafter. Yet, one of the most significant concerns with regard to enabling long-duration human spaceflight missions is development of a sustainable, reliable, and nutritious food supply that can be grown onboard the spacecraft. In the most recent decadal survey, the National Aeronautics and Space Administration (NASA) identified plant systems for long-duration life support as one of the highest priority areas of research to move human exploration beyond Low Earth Orbit (LEO) (National Academies of Sciences, Engineering, and Medicine, 2017). The species *Lemna gibba* in the duckweed family (Lemnaceae) is a very attractive option for a plant system that can be used to sustain human life onboard a spacecraft. Duckweeds have been widely studied for their wastewater-reclamation properties (El-Shafai et al., 2007; Mohedano et al., 2012; Oron, 1994; Oron et al., 1985, 1987; Ozengin & Elmaci, 2007), and are now receiving attention as a potential plant food source for space applications because of their high nutritional value. Duckweeds produce high levels of essential human micronutrients (Appenroth et al., 2017) needed to combat damage from the environmental conditions during spaceflight, including radiation exposure and its physiological consequences, such as system-wide chronic inflammation and specific effects on the ocular system (human eye). Furthermore, duckweeds are unique among non-leguminous plants in their high content of protein that contains all essential human amino acids (Appenroth et al., 1982, 2017). While soybean also has all essential human amino acids (Journey et al., 1991; Michelfelder, 2009), only a small portion of soybean biomass is edible, whereas the entire duckweed plant is consumable (Journey et al., 1991). This harvest index of 100% makes duckweed an especially attractive option for growth in space, where volume is limited and waste generation must be minimal.

This research seeks to address the question of how the nutritional quality of duckweed can be maximized to maintain astronaut health on future human spaceflight missions. To address this question, I conducted a literature review of the spaceflight environment as well as the beneficial effects of micro- and macronutrients found in duckweed (see section 3). Additionally, my thesis consisted of two data analyses that addressed the question of how specific features of plant growth environments (here light intensity) affects duckweed nutritional quality. I first analyzed data previously collected by Dr. Jared Stewart (some of which are published in Stewart et al. 2020 and some of which are unpublished) to assess how growth light intensity impacted production of the micronutrients zeaxanthin, lutein, β-carotene (pro-vitamin A), and tocopherol (vitamin E). *L. gibba*

was grown under six conditions varying in density of light particles (photons) received by a given plant area per second, i.e., the photon flux density (PFD), and resulting concentrations of the human micronutrients lutein, zeaxanthin, vitamin E (tocopherol), and pro-vitamin A (β-carotene) were determined. For the experimental portion of my thesis, I collected preliminary data on macronutrient (protein) concentration in duckweed grown under different growth light intensities. Based on the literature (see section 3), I predicted that *L. gibba* would have (i) a high protein content under most or all growth conditions used in this thesis, (ii) that the concentrations of lutein, tocopherol, and β-carotene would be maximal under low growth PFDs, but that (iii) zeaxanthin accumulation would require very high growth PFDs. Further protein analysis will be needed to confirm my preliminary protein data. Finally, based on my literature review (see section 3), I predicted that there would be no single growth condition under which all of duckweed's nutrients are maximal.

3. BACKGROUND

3.1 Human Spaceflight History and Present-Day Activities

In April 1961, Russian cosmonaut Yuri Gagarin became the first person in space when he embarked on a flight that lasted less than two hours (Harrison, 2001). Since that historic trip, more than 500 people have visited outer space. Today's human spaceflight expeditions primarily target the International Space Station (ISS), a massive laboratory that orbits our planet in LEO, just a little more than 200 miles overhead. Since the establishment of the ISS in 2000, humanity has had a continuous presence in space. Contemporary astronauts visit the station for missions that are typically six months long, but can be anywhere from three months to approximately one year in duration. The ISS is relatively large, nearly the size of a football field in length and larger than a six bedroom house in terms of habitable volume (Garcia, 2016). Although it seems far away, trips between the ISS and Earth are relatively fast and can be accomplished within hours (Barratt & Pool, 2008). The experiments conducted onboard the ISS have advanced our understanding of both the physical and biological sciences, and the continuous human presence on the ISS has expanded our understanding of how spaceflight affects human physiology. Furthermore, given its close proximity to the Earth, resupply missions to the ISS are feasible and occur on a regular basis to deliver food, clothing, science experiments, and more to the astronauts onboard the station. However, as humanity prepares to move beyond LEO, back to the Moon, and eventually to Mars,

the extended distance means that resupply will become far less feasible, and our approach to human spaceflight will need to change.

3.2 Future of Human Spaceflight

Space Policy Directive 1, which is set by the President of the United States, calls on NASA to develop a program to return humans to the Moon and eventually go to Mars (Trump, 2017). More recently, in March 2019, Vice President Pence instructed NASA to make the return to the Moon by 2024 (Chang, 2019). The Moon will serve as a testing-ground to perfect technology that will be essential for the first crewed mission to our neighboring planet, Mars. Long-duration, deep space missions such as these will occur in the very near future, but they present a wide array of new challenges beyond those experienced in human spaceflight missions to LEO. Using existing technology, it could take up to three years for astronauts to travel to Mars, explore its surface, and return to Earth (Cucinotta et al., 2013). Furthermore, existing plans for Orion, the spacecraft that will transport a crew of four to six people to Mars, indicate that the habitable volume of the vehicle will be 361 ft^3, which is only 2.64% of the size of the ISS (Garcia, 2016; Hatfield, 2006). Given the extreme distances involved in a crewed mission to Mars, resupply missions are impractical and highly unlikely to occur. This means that everything the astronauts will need for a multi-year mission, including food and water, must fit inside their spacecraft, an issue further complicated by the extremely small size of the vehicle. Moreover, astronauts must follow a nutritious diet to ensure they are healthy enough to fulfill their exploration duties upon arrival on Mars. Given the size of the spacecraft, it is unreasonable to bring food from Earth, which makes it essential to develop a reliable, renewable, and highly nutritious food source for these missions. A crewed mission to Mars has tremendous scientific potential and is certain to push the boundaries of humanity's knowledge, yet many challenges, notably those related to food and nutrition, must be addressed before such a mission becomes possible.

3.3 Medical Hazards of Human Spaceflight

The human body and its physiological functions evolved under the force of gravity. When the fundamental force of gravity is removed, as in a space environment, human physiology begins to change in an attempt to adjust to the new setting. While some changes are minor, others have serious physiological consequences for bodily functions when people return to a gravity or partial-

gravity environment. Notably, in space, astronauts' muscles and bones are no longer responsible for bearing weight and, consequently, degrade at a remarkable rate of 1-2% per month (Carmeliet & Bouillon, 1999) due to increased activity of osteoclasts (bone-degrading cells) and decreased activity of osteoblasts (bone-forming cells) (Blaber et al., 2010). Furthermore, astronauts frequently lose 20% or more of total muscle mass, dependent on muscle-fiber type, during a typical six-month-long mission (LeBlanc et al., 2000; Trappe et al., 2009). Other spaceflight-induced health consequences include fluid redistribution from the lower extremities toward the upper body (Shen & Frishman, 2019), disruption to the sensory motor system (Blaber et al., 2010), and psychological effects as a result of stress, isolation, and confinement (Palinkas, 2001). While all of these changes are serious, this thesis will not focus on the impacts of spaceflight on these specific body systems but will instead address system-wide physiological changes in the human immune system that are exacerbated in space environments (Guéguinou et al., 2009) and are known to trigger a wide range of diseases and disorders (Borchers et al., 2002; Crucian et al., 2018; Crucian & Sams, 2009).

This thesis focuses on the nutritional benefits of the duckweed species *L. gibba* and the role it could play in combating the effects of deep-space radiation and the resulting chronic inflammation and immune-system dysregulation. Space radiation has been identified by many as one of the, if not the most, significant risks to astronaut health on missions beyond LEO (Chancellor et al., 2014; Cucinotta et al., 2013; Simonsen et al., 2000). One of the most prevalent types of radiation present beyond LEO is galactic cosmic rays (GCR) composed of highly energetic particles, including protons as well as high-charge and -energy nuclei (HZE) originating from outside our solar system (Chancellor et al., 2014; Cucinotta et al., 2013). Outside of the Earth's protective magnetosphere, astronauts will face continuous background exposure to GCR (Townsend, 2005) at rates up to three times higher than during current missions (Cucinotta et al., 2013). Additionally, astronauts will experience occasional and unpredictable solar particle events (SPEs) where the sun ejects intense radioactive particles, including protons and alpha particles (Chancellor et al., 2014; Simonsen et al., 2000; Townsend, 2005). A more acute threat than that of GCR is posed by SPEs, which play a larger role in increasing astronauts' risk for developing cancer or cataracts (Hellweg & Baumstark-Khan, 2007; Townsend, 2005). Cancer is a life-threatening condition, and cataracts will significantly impact a crew's ability to complete their mission (Townsend, 2005). In space, radiation is an unavoidable threat, and, since existing protective

shielding materials are ineffective (Laiakis et al., 2007), radiation exposure and its associated health risks must be taken very seriously.

Ionizing radiation, like that found in space, has both direct and indirect impacts on human physiology. Upon absorption, radioactive particles can directly disrupt atomic structures of biological molecules or indirectly impact physiological function by generating reactive oxygen species (ROS) (Azzam et al., 2012; Baselet et al., 2019). Radiation absorption leads to the excitation and ionization of water, which results in unstable electrons that give rise to free radical species, including ROS (Azzam et al., 2012). Different ROS are formed as a result of exposure to different types of radiation and are distinguished based on their chemical nature and the amount of energy transferred by the radiation to the material with which it interacts in linear energy transfer (LET) (Azzam et al., 2012). GCR is considered high LET radiation (Chancellor et al., 2014) that produces the ROS superoxide, $O_2^{\bullet-}$ in the highest amounts (Azzam et al., 2012). The formation of ROS is intensified in highly oxygenated environments (Azzam et al., 2012), such as the 100% oxygen (O_2) pre-breathe astronauts must complete to prepare for extravehicular activity (EVA) (Smith et al., 2005, 2009; Smith & Lane, 1999). Additionally, ROS can be generated internally as a result of very intense exercise, such as an EVA, resulting in muscle fatigue and, therefore, a less productive EVA (Smith et al., 2009). Unique aspects of spaceflight, including radiation, 100% O_2 during an EVA, and intense exercise result in production of significant amounts of ROS in the body.

Low levels of ROS are actually necessary for normal cell functions and signaling pathways (Azzam et al., 2012). Furthermore, the body can form antioxidant enzymes to keep ROS levels in check (Smith & Lane, 1999). For example, moderate-intensity exercise can induce the production of important antioxidant enzymes (Belviranlı & Gökbel, 2006; Ji, 2002; Ji et al., 2006) that then play a role in recycling lipid-soluble antioxidants, such as vitamin E (Packer, 1991; see Fig. 1). While human antioxidant systems are effective in keeping ROS under control up to a certain level, antioxidant capacity can be exceeded in unique spaceflight environments such as an EVA with 100% O_2 (Smith et al., 2009) and other sources of ROS. In fact, after just 30 minutes in a 100% O_2 environment, ROS-induced oxidation of membrane lipids and other ROS-induced modifications increase (Smith et al., 2009; for details, see below). During spaceflight, the natural balance between antioxidants and oxidants becomes disrupted, and the ratio shifts towards oxidants (Manda et al., 2008).

In high amounts, ROS can lead to oxidative stress and immune dysregulation and eventually to damage to membranes, DNA, and other essential cellular structures (Azzam et al., 2012; Baselet et al., 2019; Smith & Lane, 1999; Stark, 1991; see Fig. 1). As stated above, ROS oxidize membrane lipids to lipid peroxides that, in modest amounts, serve as a protective early-warning system when the body experiences rising ROS production. Lipid-peroxidation-based gene-regulating hormones trigger the formation of antioxidant enzymes that are not produced without some ROS stress (Lei et al., 2016). The problem starts when ROS production exceeds the finite capacity of the body to produce antioxidant enzymes, which leads to accumulation of unopposed oxidants, immune-system dysregulation (also termed chronic inflammation), and damage to vital cell constituents (Demmig-Adams & Adams, 2013; Federico et al., 2007; Polutchko et al., 2015; see Fig. 1). Furthermore, ROS production is a snow-balling, or feed-forward event; cells damaged directly by radiation or ROS secrete inflammation-promoting hormones (cytokines and others) that generate more ROS in neighboring cells not originally damaged (Smith et al., 2009). ROS especially target the double bonds of a type of membrane lipid called polyunsaturated fatty acids (PUFAs) that come in the two forms: omega-3 and omega-6 fatty acids (Demmig-Adams & Adams, 2013; Stark, 1991). PUFAs are found in very high concentrations in the human eye, which is an area at particularly high risk for oxidative attacks because it is rich in oxygen (Demmig-Adams & Adams, 2013; Polutchko et al., 2015). While the effects of spaceflight on the ocular system are not very well understood, microgravity is known to impact vision and the shape of the eye (Mader et al., 2011), and radiation exposure increases astronauts' risk of developing cataracts through ROS-induced mechanisms (Blakely & Chang, 2007; Cohu et al., 2014; Cucinotta et al., 2001; Rafnsson et al., 2005; Schoenfeld et al., 2011; Smith & Zwart, 2008; Townsend, 2005). Antioxidant defense systems appear lowered in spaceflight (which could possibly be due to a lack of physical activity needed to produce the ROS that trigger antioxidant enzyme production), and oxidative damage, notably in ocular tissue, is increased (Smith et al., 2001, 2009, 2014; Smith & Zwart, 2008). The available evidence indicates that it will be crucial for astronauts exposed to dangerous cosmic radiation in space to maintain suitable nutrition, including a sufficient amount of dietary antioxidants (Cena et al., 2003; Demmig-Adams & Adams, 2013; Ma et al., 2014; Polutchko et al., 2015; see Fig. 1 and further details below).

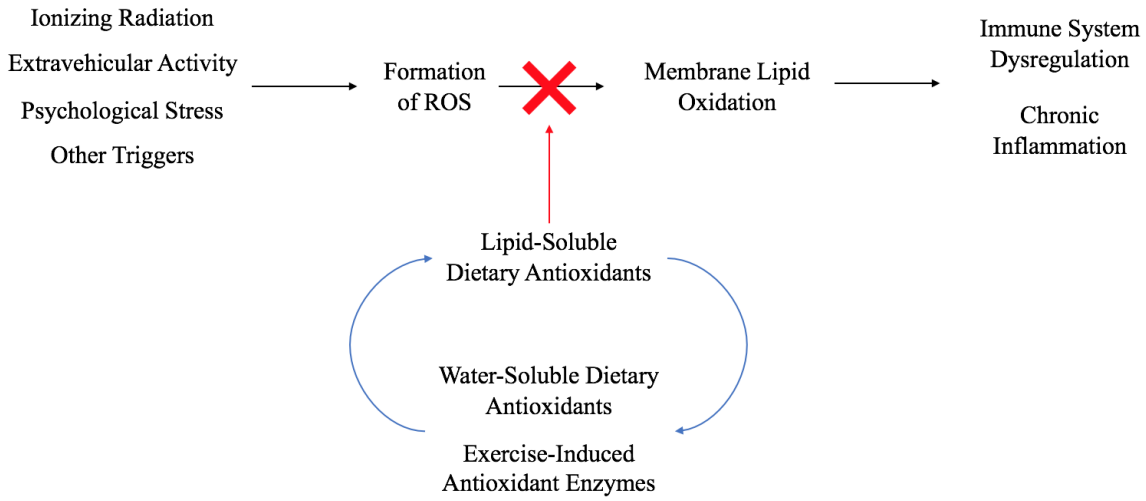

Figure 1. Schematic depiction of how chronic inflammation is triggered and opposed. Black arrows: chronic inflammation induction pathway. Red arrows: chronic inflammation prevention. Blue arrows: recycling of the lipid-soluble antioxidants by water-soluble antioxidants and exercise-induced antioxidant enzymes.

3.4 Human Nutrition in Space

Currently, astronauts have a number of food choices on the ISS. The ISS has a menu of shelf-stable, rehydratable foods (Smith & Zwart, 2008) that rotates every 8-16 days (Smith et al., 2013). Additionally, given the ISS' proximity to Earth, astronauts get shipments of fresh fruits and vegetables on a regular basis. Astronauts are not required to consume specific items for each meal but can choose from a set menu designed to fulfill nutritional requirements outlined by the World Health Organization (WHO) (Smith et al., 2013). Each week, astronauts fill out a Food Frequency Questionnaire (FFQ) to record the number of servings of each item they consumed (Smith et al., 2013). Since the nutritional value and portion size of each item on the ISS menu is known (Smith et al., 2013), the FFQ makes it very easy for scientists on the ground to determine if the astronauts are consuming the nutrients stipulated by dietary guidelines. The nutritional guidelines followed on the ISS do not currently include zeaxanthin (discussed in detail below), despite the overwhelming body of scientific evidence that describes its crucial role in maintaining health, especially in the ocular system (Bernstein et al., 2016; Demmig-Adams & Adams, 2002, 2010, 2013; Fang et al., 2002; Ma et al., 2014; Polutchko et al., 2015; SanGiovanni & Neuringer, 2012; Sies et al., 1992; Skibsted, 2018; Tapiero et al., 2004; Widomska et al., 2019; Yong et al., 2009).

Based on this evidence, the nutritional guidelines should be revised for future human spaceflight missions to provide replete nutrition. Smith et al. (2014) agree that, "astronauts in space

are generally not optimally nourished." Ever since the Apollo Era, the majority of astronauts have had a less than optimal energy intake, which is associated with body mass loss (Borchers et al., 2002; Cena et al., 2003; Crucian et al., 2018; Heer et al., 2020; Smith et al., 2005, 2013, 2014) as well as chronic inflammation and oxidative stress (Crucian et al., 2018; Heer et al., 2020), which may be exacerbated by a lack of inflammation-fighting nutrients. The closed food system in space generally provides sufficient macronutrients such as protein, carbohydrates, and fat (Smith et al., 2001, 2009), but micronutrient consumption, especially antioxidants, is of significant concern (Smith et al., 2001).

Antioxidants are crucial micronutrients for spaceflight because astronauts are subjected to multiple sources of oxidative stress, including increased exposure to ionizing radiation as well as hyperoxic conditions (with more than the typical atmospheric oxygen concentration of 21% on Earth) and excessively intense prolonged exercise during an EVA. Additionally, the ISS food system is heavily animal-based, and thus high in iron content, with excessive iron consumption also associated with increased ROS production and oxidation due to the facile transfer of a single electron from the transition metal iron to O_2 forming a superoxide (Smith et al., 2014; Weiss, 1964). Antioxidants play a crucial role in fighting oxidation, promoting proper immune-system function, and protecting the ocular system (Cena et al., 2003). The immune system, in particular, is highly influenced by nutrient consumption (Crucian et al., 2018; Heer et al., 2020). The ISS menu only provides about 60% of the total recommended intake of vitamin E (all tocopherols), important antioxidants that fight ROS and lipid peroxidation (Smith et al., 2009). Even though the amount of one type of tocopherol, γ-tocopherol, is low in space, the levels of α-tocopherol are generally thought to be sufficient during spaceflight (Smith et al., 2009). However, α-tocopherol must act in synergy with the carotenoid zeaxanthin to provide full protection of biological membranes (see separate section on zeaxanthin below). In addition, β-carotene (pro-vitamin A) has antioxidant effects and is also converted to vitamin A, which plays a role in nearly all organs, but especially the eye (Smith et al., 2014). There does not appear to be any change in β-carotene content during spaceflight, but it is possible that more will be required on deep space missions where increased exposure to ionizing radiation is likely to cause excessive oxidation throughout the body (Smith et al., 2014). A diet that is rich in antioxidants, including vitamin E and β-carotene, has been highly recommended for future long-duration human spaceflight missions (Smith & Lane, 1999). Section 3.5.1 below focuses on the carotenoids zeaxanthin and lutein that are

considered essential for eye health and other aspects of health by an overwhelming body of recent scientific evidence, but are not yet routinely considered in the daily recommended allowance.

In addition to vitamins and carotenoids, omega-3 fatty acids are essential dietary components with a role in maintaining cognitive function, decreasing chronic inflammation, and protecting the body from excess oxidation (Crucian et al., 2018; Parletta et al., 2013). In space environments and ground-based analogues, a type of omega-3 fatty acid was observed to inhibit activation of NFkB, a transcription factor that influences the cell cycle and immune-system regulators and, when continuously activated by omega-6 fatty acids, can lead to chronic inflammation (Crucian et al., 2018; Zwart et al., 2010). Another downstream effect of NFkB activation by omega-6 fatty acids is the production of osteoclasts, bone-destroying cells (Zwart et al., 2010). Omega-3 fatty acids thus play a role in counteracting the bone and muscle loss astronauts experience in space (Smith et al., 2013, 2014) as part of the dysregulation of their immune systems. Furthermore, although omega-3 fatty acids have not been studied in a controlled environment in spaceflight, astronaut consumption of fish (rich in omega-3 fatty acids) was negatively correlated with bone loss (Smith et al., 2014). No such effects were observed when fish oil supplements were consumed (Smith et al., 2014), which suggests that a whole-food-based diet can be more physiological beneficial than consuming nutrients in the form of supplements.

While seemingly attractive because of their small size, supplements do not appear to be a feasible option for long-duration spaceflight. The only supplement astronauts currently consume in space is a vitamin D supplement, as there has been a clear and consistent deficit of vitamin D consumption in space (Garrett-Bakelman et al., 2019; Smith et al., 2009). Often, supplements provide excessive amounts of nutrients, which can be toxic (Cohu et al., 2014; Smith et al., 2009, 2014). A whole-food-based diet is better for nutrient uptake by the body (Cohu et al., 2014; Smith et al., 2009) as supplements often lack the synergistic effects that result when multiple nutrients are consumed together in whole food (Smith et al., 2014). Additionally, there are psychological benefits to consuming whole food as opposed to supplements (Cena et al., 2003; Smith et al., 2009). Furthermore, the effects of long-term storage and exposure to microgravity and radiation on the stability of supplements is not well understood, and degradation could potentially lead to further nutrient deficiencies (Smith et al., 2009; Zwart et al., 2009).

Given the small size of the spacecraft that will send people to deep space, it is not feasible to stockpile all required food. Additionally, the nutritional content of food degrades when stored

over a long period of time. Cooper et al. (2017) studied 109 of the 203 available foods on the ISS and found that, when stored for three years (the length of a Mars mission), nutrient content, in particular vitamin A and vitamin C, decreased to potentially dangerously low levels. Some foods stored on the ISS for 880 days also exhibited decreased levels of β-carotene (pro-vitamin A), vitamin E, and vitamin C (Zwart et al., 2009). Due to the nutritional risks and infeasibility of storing food, it is important to develop a highly reliable, renewable, and nutritious plant food supply. The current ISS food system is rich in meats, primarily beef and chicken (Perchonok & Bourland, 2002), as opposed to fruits and vegetables (Crucian et al., 2018), and protein intake is generally sufficient (Smith et al., 2014), but the fat composition of these meats, as well as the majority of other foods on the ISS, has not been thoroughly studied (Smith et al., 2009). Adequate protein intake is important for immune-system health, including maintenance of a sufficient supply of infection-fighting, protein-based antibodies (Chandra et al., 1984) and other protein-based components of the immune system (Guadagni & Biolo, 2009; Smith et al., 2014). Additionally, protein is critical for counteracting the muscle loss astronauts experience in space (Smith et al., 2014). However, on future missions, animal protein will be less available, and vegetables will thus be a more significant source of protein (Smith et al., 2014). A higher intake of vegetables will be required because vegetables generally have less protein than meats (Smith et al., 2014); future food systems should thus include plants with exceptionally high protein content and robust growth, such as duckweed.

The decreased energy and nutrient intake associated with present-day spaceflight missions could be life-threatening on long-duration missions (Cena et al., 2003; Smith & Zwart, 2008). Furthermore, it is possible that long-duration exposure to the extreme environment of spaceflight will require an increased intake of crucial antioxidants that cannot be produced by the body, which has led to a call for further study (Smith et al., 2014; Smith & Zwart, 2008; Zwart et al., 2009). My thesis addresses the plant side of this need in seeking to understand how production of essential nutrients can be co-optimized in duckweed.

3.5 Duckweed's Unique Suitability for Human Spaceflight Applications

As humans prepare to venture into deep space for long periods of time, a regenerative, nutritious, and highly reliable food supply needs to be developed. Since crop failure will be fatal on these long-duration missions (Smith & Zwart, 2008), growth must be highly reliable. In

addition, production of the food source should require minimal space and resources, such as light and water. Even though other plants may fit these specifications, duckweed meets and exceeds all of these requirements and has many other unique qualities that make it advantageous for growth in space. Duckweed is completely edible and does not have any cytotoxic effects on human cells (Sree et al., 2019). It is a small, aquatic plant capable of growing in diverse environments, such as a water film that is only a few millimeters deep (Leng, 1999). In addition to occupying minimal volume, duckweed requires little structural support to grow, making it suitable for the gravity-free space environment. In a simulated microgravity environment, duckweed's relative growth rate was actually enhanced, and the plant remained structurally intact (Yuan & Xu, 2017). Furthermore, duckweed is one of the fastest-growing plants currently known – capable of doubling its biomass in about 1 to 3 days (Ziegler et al., 2015). This high turnover rate could provide astronauts with a continuous supply of fresh food. Additionally, duckweed thrives in environments with elevated carbon dioxide (CO_2) concentrations and is well-suited for removing CO_2 exhaled by humans from the air and supplying the O_2 humans need to breathe. When grown in an environment with CO_2 levels resembling that of the ISS, duckweed's relative growth rate further increased (Landolt & Kandeler, 1987). Duckweed's growth capabilities, especially in environments resembling space, make it an attractive option for human spaceflight purposes. In addition to being structurally suited for spaceflight, duckweed is a promising food source for astronauts because of its superb nutritional value. The duckweed plant contains a high level of high-quality proteins, fats, and micronutrients (Appenroth et al., 1982, 2017), all of which are discussed in more detail below. In terms of micronutrients, zeaxanthin and lutein are included in this thesis because they are specific, very important protectors of the eye that support visual acuity under high and low light respectively (Fig. 2). Additionally, zeaxanthin and α-tocopherol are included because of their synergistic interactions to promote system-wide membrane protection (Fig. 2). Finally, β-carotene (pro-vitamin A) is included in this analysis because it is a precursor to vitamin A that is needed as a critical component of the vision protein that allows humans to see (Fig. 2). In summary, the four micronutrients are included in this study because of their ability to either promote visual acuity or prevent vision loss, or contribute to both (Fig. 3).

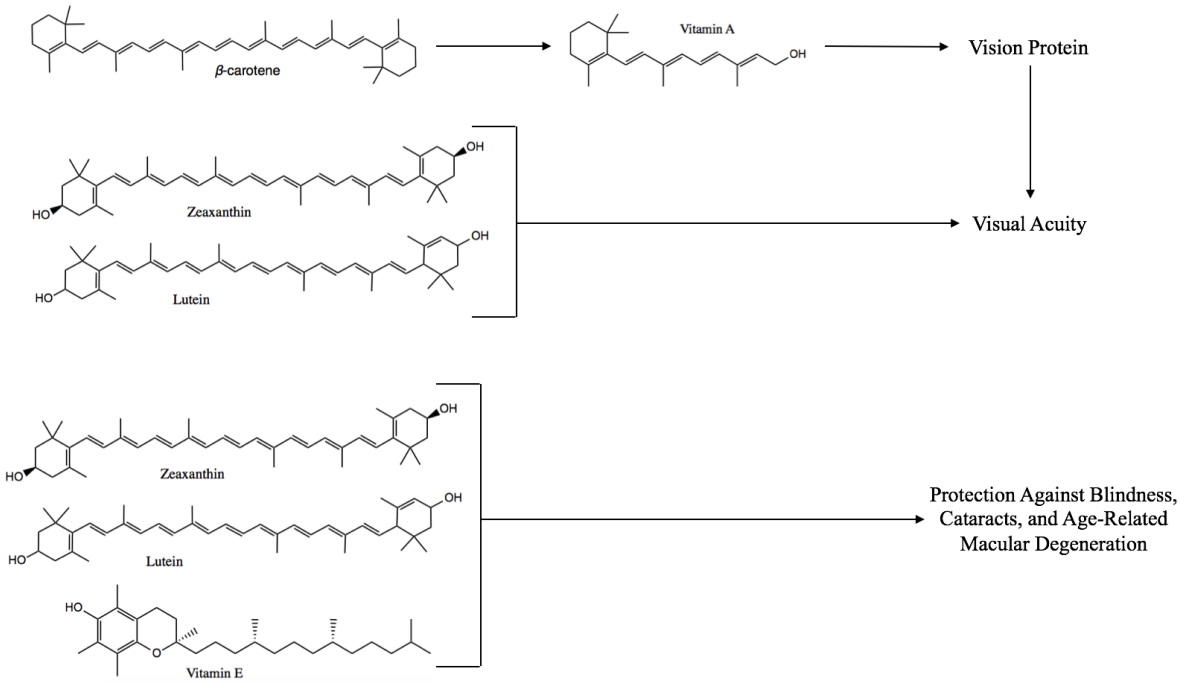

Figure 2. Schematic depiction of the dietary requirements for visual acuity and protection by carotenoids and tocopherol (vitamin E) against degenerative eye disease. Lutein and zeaxanthin are xanthophylls, a type of carotenoid that contains oxygen in its structure, whereas β-carotene is a carotenoid with an oxygen-free structure.

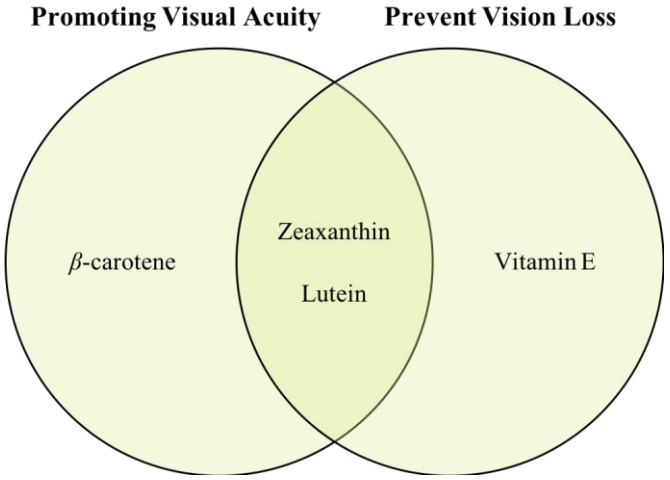

Figure 3. Venn diagram depicting the of roles of the micronutrients studied in this thesis (zeaxanthin, lutein, β-carotene, and tocopherol) in protecting eye health.

3.5.1 The Antioxidants Lutein and Zeaxanthin

There are striking similarities between the mechanisms plants employ to protect themselves from intense light and those that occur in the human eye (Polutchko et al., 2015). Two molecules, zeaxanthin and lutein, play a large role in both of these settings. Neither zeaxanthin nor lutein can

be produced by the human body, and we must obtain these essential micronutrients through the diet (Demmig-Adams & Adams, 2013). Zeaxanthin and lutein are structural isomers and are both xanthophylls, a type of oxygen-containing carotenoid (Demmig-Adams & Adams, 2013; see Fig. 2).

Zeaxanthin is a key photoprotector for plants. Zeaxanthin concentration is highest when plants are subjected to intense light conditions (Demmig-Adams & Adams, 1996). Under high light, plants absorb excess energy, which could potentially be detrimental, but zeaxanthin protects the plant by removing, or dissipating, this excess light energy as harmless heat energy (Demmig-Adams, 1990; Demmig-Adams & Adams, 1996). Lutein also plays a role, but a far less significant one, in thermal dissipation (Demmig-Adams & Adams, 2002). When light intensity decreases, zeaxanthin is quickly converted back to its precursor in order to prevent removal of the now-limited light energy that is required for photosynthesis and plant growth (Demmig-Adams & Adams, 1996). Growth of plants under very high light can lead to oxidative stress and decreased biomass production (Cohu et al., 2014). However, duckweed has unusual properties in this respect and maintains biomass production up to very high light intensities, as is shown later in this thesis (see also Stewart et al. 2020). Since zeaxanthin is only found in plants under actual exposure to intense light, plants typically have little to no zeaxanthin left when they reach today's human consumer (Cohu et al., 2014; Polutchko et al., 2015). In fact, the only naturally available foods that are rich in stable zeaxanthin are eggs and corn (Demmig-Adams & Adams, 2010). Lutein, on the other hand, is accumulated at lower light levels because it defends against a potentially hazardous excited state of chlorophyll that is not used for photosynthesis (Dall'Osto et al., 2006; Stewart et al., 2020). Therefore, lutein is easier to come by in the diet. Lutein's availability does not make up for low levels of zeaxanthin because the two serve different functions as related to the eye (see below).

The traditional Western diet is lacking in zeaxanthin even though this nutrient is essential for maintaining eye health and appears to be linked to a reduced risk, not only for eye disease, but also for cancer, heart disease, stroke, and other pro-inflammatory (immune-system-dysfunction) diseases (Mares-Perlman et al., 2002; Ribaya-Mercado & Blumberg, 2004). Dietary zeaxanthin and lutein are associated with a decreased risk of developing ophthalmic diseases, such as cataracts and age-related macular degeneration (Ma et al., 2014; SanGiovanni & Neuringer, 2012; see Fig. 2). The human eye is an oxygen-rich environment and, thus, particularly vulnerable to ROS

attacks. This is probably a reason why high amounts of zeaxanthin, lutein, vitamin E, and omega-3 fatty acids are found in the eye (Polutchko et al., 2015). Both zeaxanthin and lutein play significant roles in eye protection, but zeaxanthin is the primary photoprotector in the human eye, just as it is in the plant (Cohu et al., 2014; Polutchko et al., 2015). Zeaxanthin is more preferentially accumulated in the part of the eye hit by intense light and cannot be replaced by lutein (Cohu et al., 2014; Polutchko et al., 2015). Specifically, zeaxanthin is found in high concentrations in the central region of the retina that is responsible for visual acuity in high light (Polutchko et al., 2015). On the other hand, lutein is involved in low-light vision and found in higher concentrations in the outer edges of the retina where light is less intense (Polutchko et al., 2015). There is some evidence that zeaxanthin accumulation in the eye differs among people with different eye colors (Hammond et al., 1996), which suggests that genetics may play a role in possible differential requirements among people for zeaxanthin.

Zeaxanthin and lutein have lipid-soluble nonpolar center portions and polar ends that fit well into biological membranes (which also have nonpolar center portions and polar outer portions) and are thus uniquely suited to protect biological membranes from lipid peroxidation by ROS as well as optimize membrane fluidity (Tapiero et al., 2004; Woodall et al., 1997). A molecule's ability to detoxify ROS may furthermore increase with its number of double bonds – zeaxanthin has 11 and lutein has 10, which makes them both good candidates for ROS detoxification (Skibsted, 2018). However, other mechanisms may contribute as well and need to be further elucidated (Demmig-Adams & Adams, 2013). Zeaxanthin prevents programmed cell death of photoreceptor cells (which is the cause of macular degeneration, or blindness), inhibits membrane-lipid oxidation, and fights chronic inflammation (Demmig-Adams & Adams, 2010, 2013).

Zeaxanthin also acts synergistically with other antioxidants, including vitamin E (Fig. 2) and omega-3 fatty acids, to protect the eye from intense light (Demmig-Adams & Adams, 2013). In a study where zeaxanthin acted alone, its effect in limiting lipid peroxidation was far less significant than when other antioxidants, such as α-tocopherol or vitamin C, were also present (Wrona et al., 2004). When zeaxanthin acted alone, its photoprotective effect was far less significant than when the other antioxidants were also present (Wrona et al., 2004). The concomitant presence of α-tocopherol and the water-soluble vitamin C slowed down the rate of zeaxanthin depletion, thus increasing zeaxanthin's protective effects (Wrona et al., 2003, 2004).

Single supplements of zeaxanthin and lutein do not have the same benefit as dietary consumption with whole food, where additional nutrients act synergistically with zeaxanthin and lutein (Demmig-Adams & Adams, 2010). Duckweed can have high concentrations of zeaxanthin, lutein, vitamin E and other synergistically acting antioxidants, making it an attractive dietary option to promote eye health. However, the effect of growth conditions on nutrient concentration – and potential differential effects on different nutrients – remains to be explored, as is addressed in this thesis

3.5.2 The Antioxidant Vitamin E (Tocopherol)

The antioxidant vitamin E (tocopherol) is found in several forms, with 90% of tocopherol in the human body represented by α-tocopherol (Smith et al., 2009). Exposure to radiation in space compromises antioxidant activity (Kennedy et al., 2007). As stated above, a lack of antioxidants leads to over-stimulation and derailment of the immune system, programmed cell death, and pro-inflammatory diseases (Polutchko et al., 2015; see Fig. 1), but this can be mitigated by a diet rich in antioxidants such as vitamin E (Kennedy et al., 2007; Schneider, 2005; see Fig. 2). Additionally, vitamin E apparently plays a role in mitigating bone and muscle loss experienced by astronauts in spaceflight (Smith et al., 2009). Duckweed is rich in tocopherol (Appenroth et al., 2017).

Tocopherol plays a significant role in protecting the human body from ROS (Mocchegiani et al., 2014; Peh et al., 2016; Smith et al., 2009). Vitamin E is crucial to the protection of PUFAs (including omega-3 fatty acids) in cell membranes from ROS (Smith et al., 2009; Traber & Atkinson, 2007). This mechanism decreases the amount of lipid peroxidation products, such as prostaglandin hormones (Smith et al., 2014). However, this protective action of donating an electron to ROS converts tocopherol into a radical, which can be recycled (reduced) by the water-soluble antioxidant vitamin C to restore the supply of vitamin E (Demmig-Adams & Adams, 2013; Smith et al., 2009; Tapiero et al., 2004; see Fig. 1, 4).

While tocopherols are generally more effective at scavenging ROS than carotenoids such as zeaxanthin and β-carotene (Skibsted, 2018), vitamin E also works synergistically with zeaxanthin to prevent lipid peroxidation (Demmig-Adams & Adams, 2013). In fact, α-tocopherol alone was not sufficient to protect membranes from lipid peroxidation but, when combined with zeaxanthin, achieved enhanced protection of lipid membranes from oxidation (Wrona et al., 2003).

It was proposed that vitamin E and zeaxanthin act through complementary mechanisms in detoxifying different types of reactive species (Wrona et al., 2003).

Much like zeaxanthin and lutein, single supplements of vitamin E do not have the same effects as dietary consumption with food. When consumed in whole food, nutrients such as vitamin E are more effectively taken up by the body and are able to act synergistically with other ingredients (Demmig-Adams & Adams, 2010, 2013; Polutchko et al., 2015).

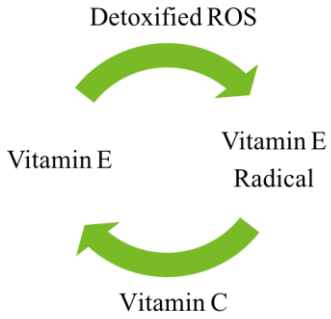

Figure 4. Schematic depiction of membrane-soluble tocopherol (vitamin E) detoxifying ROS and being regenerated by water-soluble vitamin C at the interface between biological membranes (where ROS arise) and the water-based cell fluid.

3.5.3 Vision Pigment and Antioxidant Pro-Vitamin A (β-carotene)

β-carotene is another antioxidant that humans cannot produce and that must be consumed in the diet. Unlike the xanthophylls lutein and zeaxanthin, *β*-carotene is a carotenoid that does not contain oxygen in its structure (Fig. 2) Again, duckweed is a good dietary source of *β*-carotene. *β*-carotene, also known as pro-vitamin A, is an oxygen-free carotenoid that can be cleaved to produce two molecules of vitamin A (Polutchko et al., 2015; see Fig. 2, 5). Vitamin A, a lipid-soluble vitamin (Tran & Demmig-Adams, 2007), is converted to retinal, a part of a vision protein that underlies the vision process itself (Cohu et al., 2014; Demmig-Adams & Adams, 2013; Polutchko et al., 2015). Vitamin A deficiency leads to blindness (Demmig-Adams & Adams, 2013; Polutchko et al., 2015; see Fig. 2). Additionally, vitamin A plays a role in regulating the human immune response (Demmig-Adams & Adams, 2013) and has either direct or indirect effects on nearly every organ (Smith et al., 2009).

Aside from being a precursor to vitamin A, *β*-carotene itself plays a significant role in the body, as it can also protect lipid membranes from oxidation. Carotenoids, such as *β*-carotene can

detoxify ROS and other radicals (Bernstein et al., 2016; Skibsted, 2018). *β*-carotene is nonpolar and detoxifies ROS in the hydrophobic part of the lipid membrane (Tapiero et al., 2004). Like the other micronutrients discussed above, *β*-carotene supplements often have excessively high doses and, thus, consumption via a whole-food-based diet is preferable (Tapiero et al., 2004).

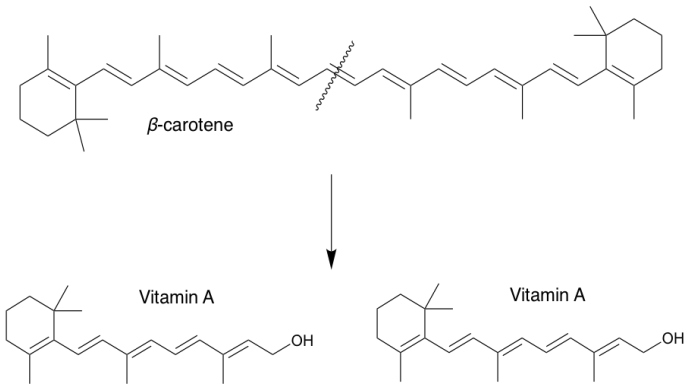

Figure 5. β-carotene is cleaved to produce two molecules of vitamin A, which plays a role in the vision process in the eye and, as a regulator of the immune response, throughout the body.

3.5.4 Protein

Protein is a macronutrient and an essential component of the human diet as it plays many important physiological roles, including muscle and bone growth, immune function, metabolism, endocrine function, fluid regulation, cardiovascular function, neurotransmitter synthesis, and many more (Castaneda et al., 1995; World Health Organization et al., 2007; Wu, 2016). Proteins are composed of amino acids, of which there are 20. Some of these amino acids (essential amino acids) cannot be synthesized by the human body and thus must be consumed in the diet. Duckweed contains all of these essential human amino acids that people need (Appenroth et al., 1982, 2017). Duckweed's protein composition more than meets WHO requirements (Appenroth et al., 1982, 2017). In contrast to legumes like soybean that also contain all essential amino acids, duckweed is 100% edible (harvest index of 100%), which makes duckweed more attractive for the spaceflight environment where volume and resources are limited and need to be used efficiently to produce as much high-quality food as possible. In addition to producing high-quality protein, duckweed also produces a high quantity of protein - up to 40% of the plant dry mass under some conditions (Appenroth et al., 2017). Duckweed's high protein content resembles legumes like soybean (Journey et al., 1991). Duckweed has such a high protein content because it is able to convert

nitrogen compounds, such as ammonia, into protein that can then be stored by the whole plant and not just the seeds (as in legumes) (El-Shafai et al., 2007; Mohedano et al., 2012; Oron, 1994; Oron et al., 1985, 1987; see Fig. 6). Like legumes, duckweed has a symbiosis with *Rhizobium* bacterium (*Rhizobium lemnae;* Kittiwongwattana & Thawai, 2014). These bacteria fix elemental atmospheric nitrogen (N_2) into reduced nitrogen, ammonium, and amino acids (Zahran, 1999). This feature presumably also contributes to the fact that duckweed, like legumes, accumulates a high amount of protein (Zahran, 1999). In turn, the ability to accumulate vegetative storage protein (everywhere in the plant) may contribute to duckweed's ability to take up nitrogen-based nutrients from the water it floats on (Oron et al., 1987). This propensity makes duckweed attractive for spaceflight applications – because it can generate protein from wastewater. Duckweed is widely used to treat wastewater because of its ability to effectively accumulate high levels of various nitrogen compounds (El-Shafai et al., 2007; Hammouda et al., 1995; Oron, 1994; Oron et al., 1985, 1987; Ozengin & Elmaci, 2007; see Fig. 6). In the context of space, duckweed could, therefore, also be used to produce high-quality protein from human waste. Since protein, in particular vegetable protein, will be an essential component of astronauts' diet on long-duration missions, duckweed's high protein content with all essential human amino acids makes the plant a very attractive option for a food source in space.

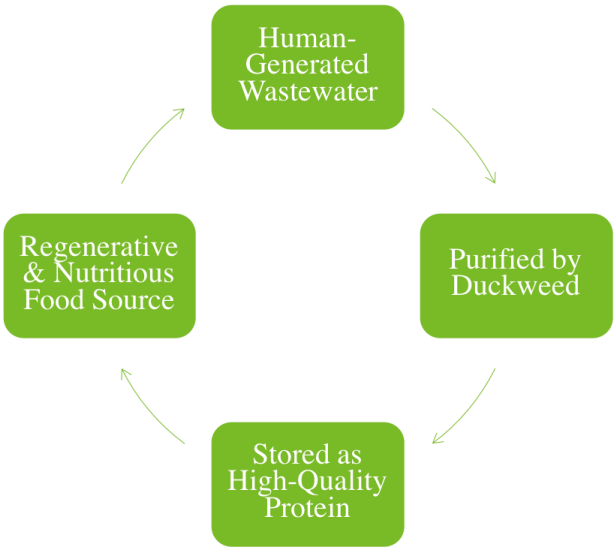

Figure 6. Schematic depiction of how duckweed reclaims wastewater by converting nitrogen-based waste to high-quality protein.

3.5.5 Fat Composition

The human body requires both omega-3 and omega-6 fatty acids from dietary sources, and these fatty acids become components of cell membranes and precursors of a large class of immune-regulatory hormones (Demmig-Adams & Adams, 2010; Kaur et al., 2014; Saini & Keum, 2018; Surette, 2008). Since omega-6 and omega-3-fatty-acid-derived hormones have immune-response-initiating and immune-response-terminating effects, respectively, it is important that these PUFAs be in an appropriate ratio (Fig. 7). The typical Western diet has a ratio of omega-6 to omega-3 fatty acids of between 15 and 20 to 1, which is excessively high (Innes & Calder, 2018; Simopoulos, 2008). Ideally, this ratio should be considerably less than 10 to 1 (Simopoulos, 2003, 2004, 2008). A high ratio of omega-6 fatty acids to omega-3 fatty acids is dangerous because it is associated with promoting chronic pro-inflammatory diseases including cardiovascular disease, diabetes, cancer, and many others (Appenroth et al., 2017; Calder et al., 2011; Innes & Calder, 2018; Saini & Keum, 2018; see Fig. 7). Omega-3 and omega-6 fatty acids play antagonistic roles in regulating genes that control cell death, cell division, and, as stated above, the immune system (Demmig-Adams & Adams, 2013; Khandelwal et al., 2013; Polutchko et al., 2015; Saini & Keum, 2018; see Fig. 7). The ROS generated by exposure to ionizing radiation target and oxidize PUFAs, including both omega-6 and omega-3 fatty acids in cell membranes (Bernstein et al., 2016). Gene regulators produced from oxidation products of omega-6 fatty acids trigger inflammation and cell death (Adams et al., 2016; Demmig-Adams & Adams, 2010; Innes & Calder, 2018; Khandelwal et al., 2013; Polutchko et al., 2015; Simopoulos, 2004). On the other hand, hormone-like messengers derived from omega-3 oxidation prevent cell death, stop inflammation, and slow down cell division (Adams et al., 2016; Calder et al., 2011; Demmig-Adams & Adams, 2010; Kaur et al., 2014; Polutchko et al., 2015; Simopoulos, 2002, 2003). Omega-3 fatty acids thus work synergistically with the antioxidants discussed above to protect against chronic inflammation and the effects of intense light in the eye (Demmig-Adams & Adams, 2002; Polutchko et al., 2015; Rasmussen & Johnson, 2013; SanGiovanni & Chew, 2005). In addition to the antioxidants discussed above, plants produce omega-3 fatty acids to protect themselves from potentially harmful radiation (Nishiuchi & Iba, 1998). Duckweed is ideal for human nutrition because it is rich in the anti-inflammatory omega-3 fatty acids, resulting in a low ratio of omega-6 to omega-3 fatty acids (Appenroth et al., 2017).

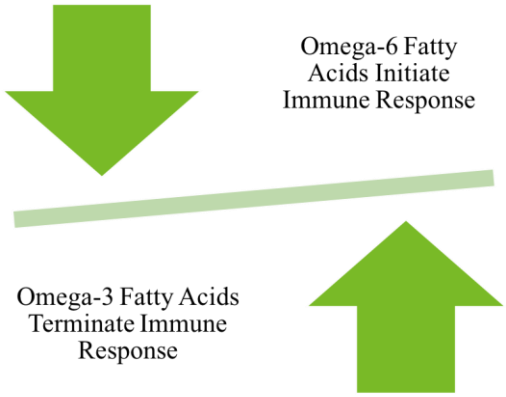

Figure 7. When oxidized by ROS, omega-3 fatty acids and omega-6 fatty acids play antagonistic roles in the immune response.

4. METHODS

4.1 Plant Growth Conditions

The duckweed species *Lemna gibba* L. used in this thesis was obtained from Rutgers Duckweed Stock Cooperative (https://rduckweed.org) and was grown as described in Stewart et al. (2020) in 1000 ml of Schenk and Hildebrandt Medium (bioWORLD, Dublin, OH, USA) in PYREX Crystallizing Dishes (Corning Inc., Corning, NY, USA). A stock culture of duckweed was maintained in a growth chamber under the constant low PFD of 50 μmol photons m^{-2} s^{-1}. Experimental samples were then taken from the stock and grown under a wide range of continuous light (24 hours a day), including 50, 100, 200, 500, 700, and 1000 μmol photons m^{-2} s^{-1} for seven days each (Fig. 8). These light intensities allowed evaluation of duckweed's production of micronutrients across a wide range of growth light environments up to a total number of photons received per day (at 1000 μmol photons m^{-2} s^{-1}) that exceeds the total number of photons received by plants on the brightest longest day of the year. The plants shown in Figure 8 (from Stewart et al. 2020) consisted of small colonies of one or more mother fronds with two or more daughter fronds each. While fronds look like leaves, they represent individual plants that divide into identical clones.

The duckweed plants I used for the micronutrient analysis in this thesis had been grown and assayed for zeaxanthin, lutein, β-carotene, and tocopherol by Dr. Jared Stewart. Zeaxanthin, lutein, and β-carotene concentrations per plant for samples grown under 100, 200, 500, and 700 μmol photons m^{-2} s^{-1} are from Stewart et al. (2020). The zeaxanthin, lutein, and β-carotene per area

data for fronds grown under 50 and 1000 μmol photons m^{-2} s^{-1} are unpublished data collected by Dr. Stewart. I used the data collected by Dr. Jared Stewart to express zeaxanthin, lutein, and β-carotene concentration per dry weight as well as per photons received by the plant (light-use efficiency, or LUE) for samples grown under 50, 100, 200, 500, 700, and 1000 μmol photons m^{-2} s^{-1}. My thesis additionally includes expression of vitamin E (tocopherol) per area, dry weight, and photons received (LUE) for samples grown under 50, 100, 200, 500, 700, and 1000 μmol photons m^{-2} s^{-1}. The vitamin E assays were conducted by Dr. Jared Stewart, and I analyzed the resulting data on the latter three reference bases.

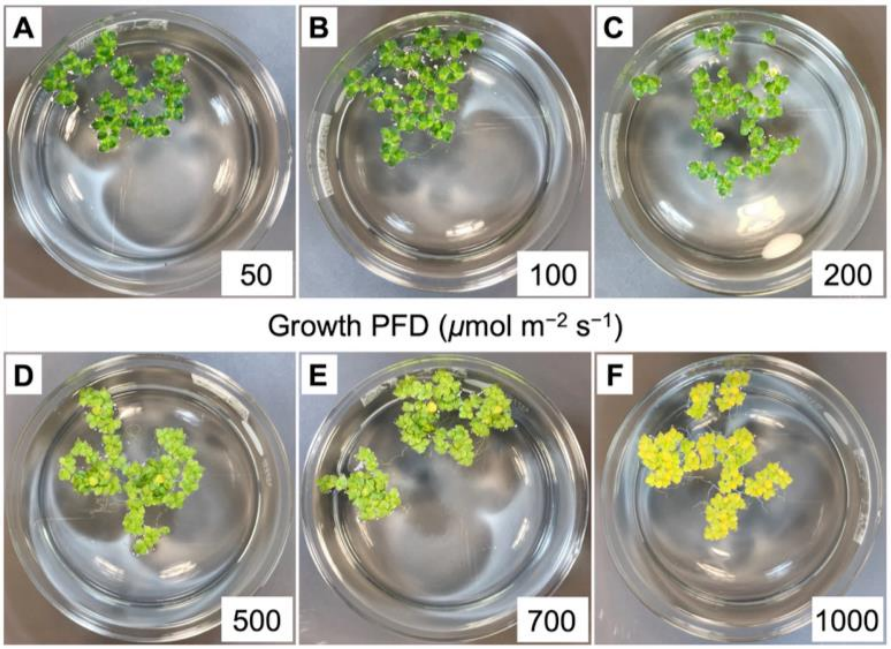

Figure 8. Images of duckweed frond growth across the six different growth light intensities (photon flux densities, or PFDs of 50, 100, 200, 500, 700, and 1000). Each dish was seeded with approximately 20 little leaves (fronds) and allowed to grow for 4 days under the same PFD, after which the photos were taken. Images B to E are from Stewart et al. (2020) and A and F are unpublished data.

4.2 Carotenoid and Tocopherol Analysis

Following plant growth under different light intensities, Dr. Jared Stewart harvested and froze samples in liquid nitrogen for subsequent analysis of the carotenoids zeaxanthin, lutein, and β-carotene (pro-vitamin A), as well as vitamin E (α-tocopherol) as described in Stewart et al. (2015) using high performance liquid chromatography (HPLC). Lutein and β-carotene were measured in fronds frozen immediately after harvesting plants as well as in fronds frozen after a

30-minute recovery period during which the plants were removed from their respective growth PFDs and maintained in low light at room temperature. Since the amount of lutein and β-carotene, as expected, did not differ significantly before and after recovery, the two time points were averaged for these two carotenoids. Vitamin E was only measured following a 30-minute recovery period. As discussed in section 3.5.1 of this thesis, when plants are no longer subjected to intense light they quickly remove zeaxanthin to avoid dissipating light that could be used in photosynthesis. In order to determine how this unique mechanism impacted zeaxanthin concentration, zeaxanthin was also measured separately in fronds frozen immediately after harvesting and those frozen after a 30-minute recovery period in low light at room temperature, and these data are analyzed and displayed separately in this thesis.

While leaf pigments were reported in Stewart et al. (2020) in units of μmol per leaf area, I converted these essential human micronutrients to units more relevant to human consumption, in particular, concentration in units of mg per frond area, mg per dry weight, and production efficiency per mol of photons received, or the light-use efficiency (LUE) of micronutrient production. The dry biomass data I used were from Stewart et al. (2020). Furthermore, I added vitamin E (tocopherol), which was not included in Stewart et al. (2020), to the consideration of human micronutrients. Expression of these human micronutrients in dry-weight reference frames is relevant to human spaceflight, as it allows for comparison of plant productivity to nutritional quality for astronauts.

Additionally, following the calculations outlined in Stewart et al. (2020), I determined the light-use efficiency (LUE) of dry biomass, zeaxanthin, lutein, β-carotene, and tocopherol production. LUE can be defined as the amount of production per the mol of photons received. The photons received (intercepted by fronds), a constant value, was reported in Stewart et al. (2020). Then, using measurements of initial and final frond area, I determined the initial and final amount of dry biomass, zeaxanthin, lutein, β-carotene, and tocopherol. I then incorporated these values and the number of photons received (from Stewart et al. 2020) into the following equation to determine LUE for these substances:

$$\boldsymbol{Light\ use\ efficiency} = \frac{(\boldsymbol{FA_t - FA_0})}{\boldsymbol{Photons\ received\ (t)}}$$

where FA_t is frond area after four days of growth; FA_0 is frond area on day 0; t is 4 days

4.3 Protein Content

The protein content of the duckweed samples was analyzed via the Lowry assay, which is recognized for its simplicity and accuracy (Lindeboom & Wanasundara, 2007; Lowry et al., 1951; Markwell et al., 1978). Dr. Marina López-Pozo grew the duckweed samples used for protein analysis under growth light intensities of 50, 200, and 1000 μmol photons m^{-2} s^{-1}. I performed the protein assay (described below) and conducted analyses to determine protein concentration on a per area basis. After extensive work with a method adapted from Lindeboom and Wanasundara (2007), I switched and used a protocol provided in the Total Protein Kit, Micro Lowry, Peterson's Modification for my final analyses (Sigma-Aldrich, Saint Louis, MO, USA; https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Bulletin/tp0300bul.pdf). This protein assay kit uses a slightly modified version of the original Lowry method that allows for rapid recovery of proteins and minimizes potential interferences of phenolics with protein detection (Peterson, 1977). Protein concentration was ascertained spectrophotometrically (Beckman DU 640 Spectrophotometer) as absorbance levels that were converted to protein levels using a standard calibration curve based on bovine serum albumin (BSA), a common protein standard. Deionized water (DI H_2O) was used to dilute BSA to known concentrations of 10, 20, 50, 80, 150, and 180 μg/ml. Duckweed samples frozen in liquid nitrogen were removed from the liquid nitrogen, and three fronds were ground using a mortar and pestle. These crushed duckweed samples were combined with 1 ml of DI H_2O, added to a microcentrifuge tube, and mixed well with a vortexer. Subsequently, duckweed samples were centrifuged for 10 minutes at 10,000 rotations per minute (rpm). The resulting supernatant contained soluble protein, and the pellet contained insoluble frond components. The supernatant was decanted into another microcentrifuge tube, and the pellet was discarded. Subsequently, 0.1 ml of deoxycholate (DOC), and, after vortexing and incubation for another 10 minutes, 0.1 ml of trichloroacetic acid (TCA), were added. This was followed by vortexing and centrifuging for 10 minutes at 10,000 rpm in order to precipitate the soluble proteins, while minimizing any possible interference from the chemicals

used in the original Lowry procedure (Lowry et al., 1951; Peterson, 1977). Following centrifugation, all relevant (soluble) proteins were in the pellet; the supernatant was thus decanted and discarded; each pellet was dissolved in 1 ml of the Lowry Reagent; and the resulting solution was transferred to a cuvette. To ensure no material was left in the microcentrifuge tubes, tubes were rinsed with 1 ml of DI H_2O, and the resulting solution was added to the cuvettes and mixed thoroughly. Solutions were kept at room temperature for 20 minutes to allow the Lowry Reagent to facilitate a reaction of peptide bonds of the proteins with copper ions of the reagent. Next, 0.5 ml of the Folin & Ciocalteu's Phenol Reagent (FCR) was added to each cuvette and mixed with a pipette. FCR oxidizes peptide bonds and undergoes reduction itself, leading to a change in the color of the solution. A darker color corresponds to a higher protein concentration. The color was allowed to develop for 30 minutes at room temperature. After 30 minutes, absorbance levels were read spectrophotometrically at 660 nm. The absorbance levels measured for the BSA standards were used to construct a standard calibration curve (Fig. 9). The program Microsoft Excel was used to determine the equation for a line of best fit for the standard curve. Absorbance levels of duckweed samples were entered into this equation as "y" values, and the protein concentration of these samples was calculated as "x." Previously collected frond dry mass and area data were used to obtain protein level as a percent of frond dry mass.

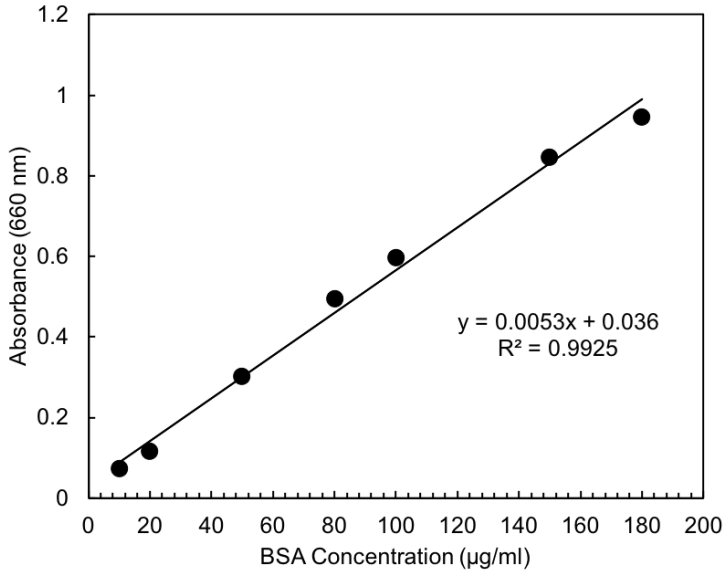

Figure 9. BSA calibration curve for protein content determination with the Total Protein Kit Micro Lowry, Peterson's Modification. BSA concentration was expressed in units of µg/ml and absorbance measured at 660nm.

4.4 Statistical Analysis

Statistically significant differences among growth PFDs were determined via analysis of variance (one-way ANOVA) and post-hoc Tukey–Kramer test for Honestly Significant Differences (HSD). One-way ANOVA analyses were conducted using Microsoft Excel. I then conducted the Tukey-Kramer HSD tests using the appropriate equations in a Microsoft Excel spreadsheet. The sample size for experiments using 50 μmol photons m^{-2} s^{-1} was 4 dishes. The sample size for experiments grown under 100, 200, 500, 700, and 1000 μmol photons m^{-2} s^{-1} was 3 dishes each.

5. RESULTS

5.1 Frond Area and Dry Weight Production

Following an experimental period of 4 days of growth under 6 different PFDs, duckweed samples were analyzed to assess which growth conditions yielded maximal area and biomass production in the context of light energy input. As shown in Figure 10, area production of duckweed fronds was relatively constant across the wide range of growth PFDs. Figure 10 displays frond area production per mol photons, i.e., the light-use efficiency (LUE) of area production, which declined precipitously with increasing growth PFD (Fig. 10A). A similar, but slightly less pronounced, drop was observed for LUE of frond dry biomass production (Fig. 10B). The difference in the degree of the decline of LUE for frond area versus frond biomass production can be attributed to the fact that the ratio of frond dry biomass per leaf area more than doubled with increasing growth PFD (Fig. 10C).

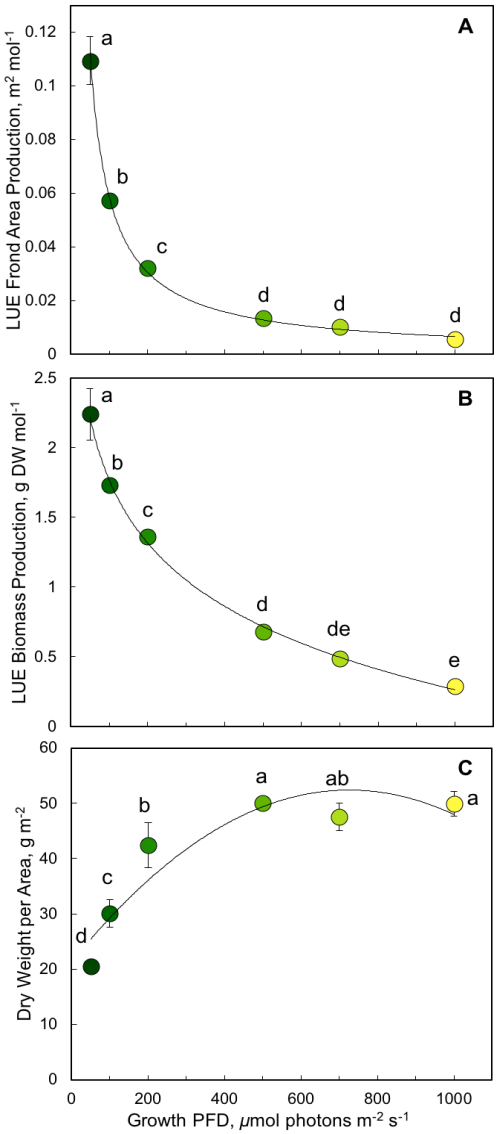

Figure 10. Light use efficiency of (A) duckweed frond area production and of (B) dry biomass production as well as (C) the ratio of dry weight per frond area for fronds grown under a range of growth PFDs. Data on area production are from Stewart et al. (2020). Data on dry biomass per area were provided by Dr. J.J. Stewart and C. M. Escobar. Lower-case letters signify significant differences at $p < 0.05$.

5.2 Lutein, β-carotene and Tocopherol Production

Production of three carotenoids, lutein, *β*-carotene (pro-vitamin A), and zeaxanthin as well as vitamin E (tocopherol) was also expressed on the basis of different reference frames, i.e., frond area, frond biomass, and as LUE of nutrient production relative to the amount of light energy required.

Lutein concentration per frond area peaked at 200 μmol photons m^{-2} s^{-1} (Fig. 11A), while lutein concentration per frond dry weight was maximal at the lowest growth PFD of 50 μmol photons m^{-2} s^{-1} (Fig. 11B). Over the course of 4 experimental growth days at each growth PFD, only one amount of lutein was produced with a single LUE. However, this lutein content can be expressed relative to the area production over the 4 days (which was rather constant across all growth PFDs) or relative to the dry biomass production over the 4 days. Dry biomass increased with increasing growth PFD, which made the lutein per dry weight curve (Fig. 11B) drop more than the lutein per area curve (Fig. 11A) as growth PFD increased. Furthermore, LUE of lutein production was highest at low growth PFDs and quickly decreased as light intensity increased (Fig. 11C).

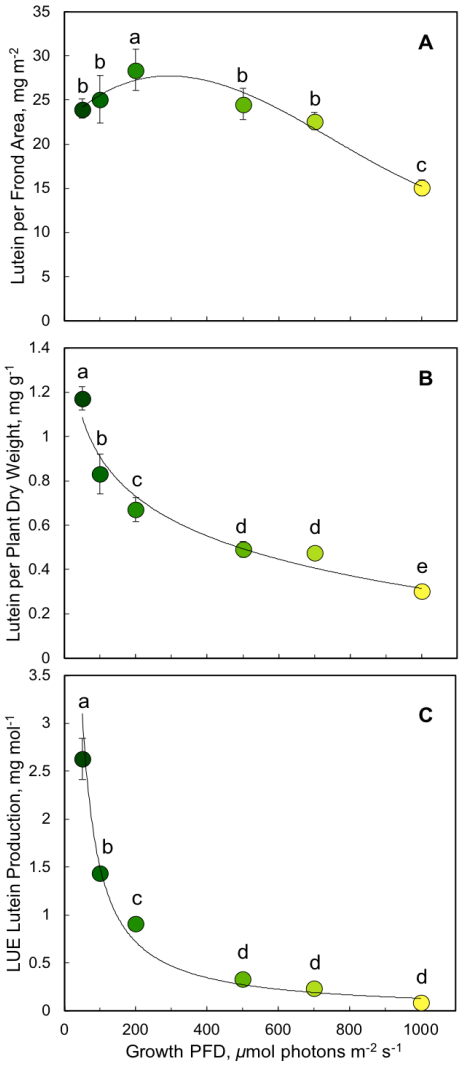

Figure 11. Production of lutein (A) per area and (B) per dry weight as well as (C) light-use efficiency of lutein production by duckweed fronds grown under a range of growth PFDs. Data on lutein levels in µmol per frond area are from Stewart et al. (2020). Lower-case letters signify significant differences at $p < 0.05$.

β-carotene concentration per frond area peaked under the growth PFD of 100 *µ*mol photons m^{-2} s^{-1} (Fig. 12A). Much like lutein, the concentration of *β*-carotene per plant dry weight reached its maximum level under the lowest growth PFD of 50 *µ*mol photons m^{-2} s^{-1} (Fig. 12B). LUE of *β*-carotene production was also highest at low growth PFDs and decreased as the light became more intense (Fig. 12C).

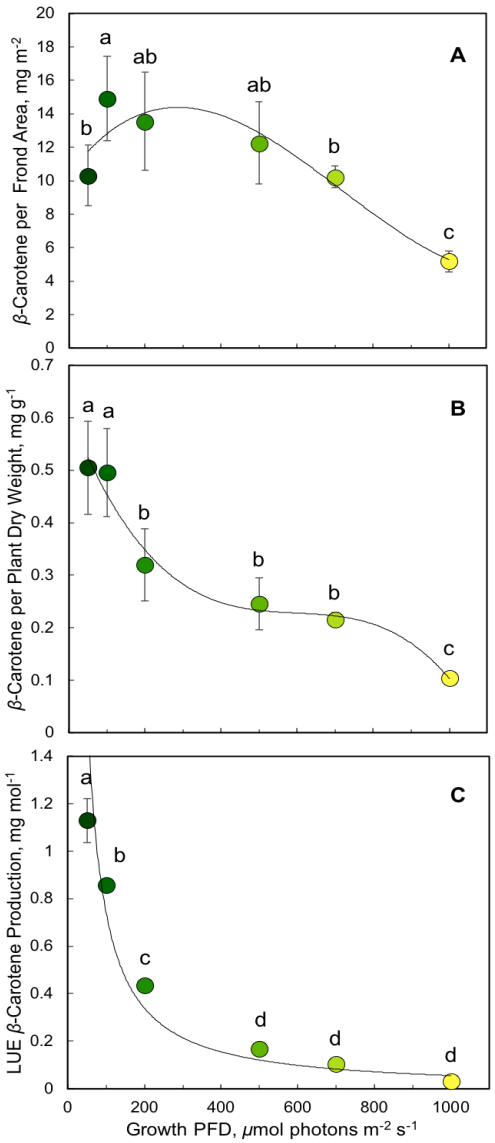

Figure 12. Production of β-carotene (A) per area and (B) per dry biomass as well as (C) light-use efficiency of β-carotene production by duckweed fronds grown under a range of growth PFDs. Data on β-carotene levels in μmol per frond area are from Stewart et al. (2020). Lower-case letters signify significant differences at $p < 0.05$.

Production of α-tocopherol under different growth PFDs followed a similar pattern as that of lutein and β-carotene. The concentration of α-tocopherol per frond area initially rose with increasing growth PFD but declined when grown under growth PFDs greater than 200 μmol photons m^{-2} s^{-1} (Fig. 13A). Like lutein and β-carotene, α-tocopherol concentration per plant dry weight was maximal at the lowest growth PFD of 50 μmol photons m^{-2} s^{-1} and began to drop as growth PFD increased (Fig. 13B). Additionally, LUE of α-tocopherol concentration decreased as growth light intensity increased (Fig. 13C).

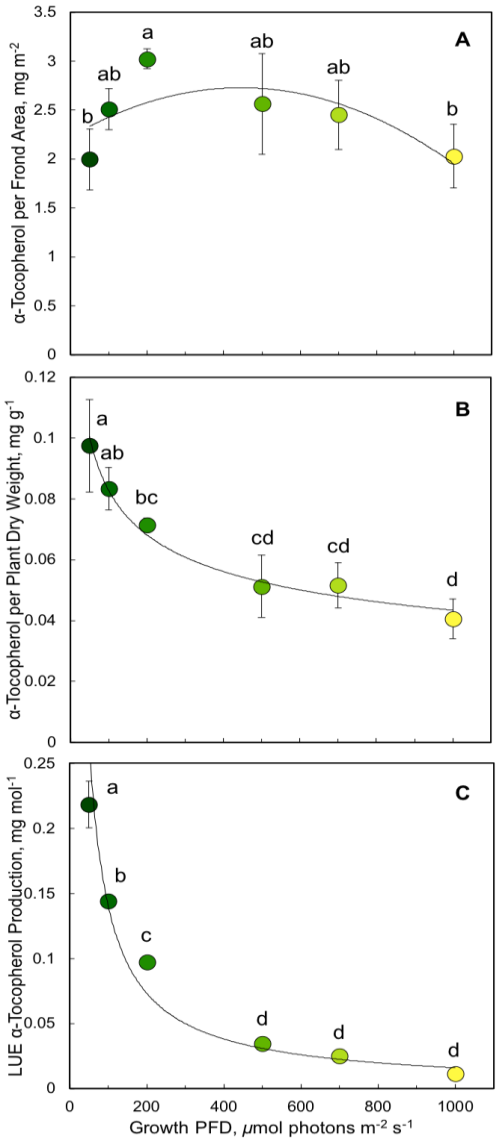

Figure 13. Production of α-tocopherol (A) per area and (B) per dry biomass as well as (C) light-use efficiency of α-tocopherol production by duckweed fronds grown under a range of growth PFDs. Data on tocopherol levels were provided by Dr. J.J. Stewart. Lower-case letters signify significant differences at $p < 0.05$.

5.3 Zeaxanthin Production

Zeaxanthin production followed a very different trend than either lutein, *β*-carotene, or *α*-tocopherol (Fig. 14). For both samples frozen immediately upon harvest (Fig. 14A) and samples subjected to a 30-minute recovery period in low light at room temperature before freezing (Fig. 14B), zeaxanthin concentration per frond area was highest at the highest growth PFD of 1000 μmol photons m^{-2} s^{-1}. In addition, zeaxanthin concentration per frond area was 3.8 mg m^{-2} lower after

recovery than immediately after harvest (Fig. 14A, B). Similar to the results for zeaxanthin on a frond area basis, zeaxanthin per plant dry weight increased with increasing growth PFD (Fig. 14C, D). After recovery, zeaxanthin concentration per dry weight was 0.08 mg g^{-1} lower than immediately after harvest for the highest growth PFD. The growth light dependency of LUE of zeaxanthin production was also affected by recovery. LUE of zeaxanthin content as assessed immediately after harvest increased quickly with increasing growth PFD, peaked at 500 μmol photons m^{-2} s^{-1}, and then began to drop as growth PFD increased further, resulting in an arc-shaped graph of the PFD dependency of LUE (Fig. 14E). On the other hand, LUE of zeaxanthin content as assessed after recovery increased more steadily, was maximal at the highest growth PFD of 1000 μmol photons m^{-2} s^{-1}, and formed a plateau-shaped graph (Fig. 14F). As was the case for zeaxanthin concentration on a leaf area and leaf dry biomass basis, LUE of zeaxanthin production was lower at each respective growth PFD after the recovery period compared to immediately after harvest.

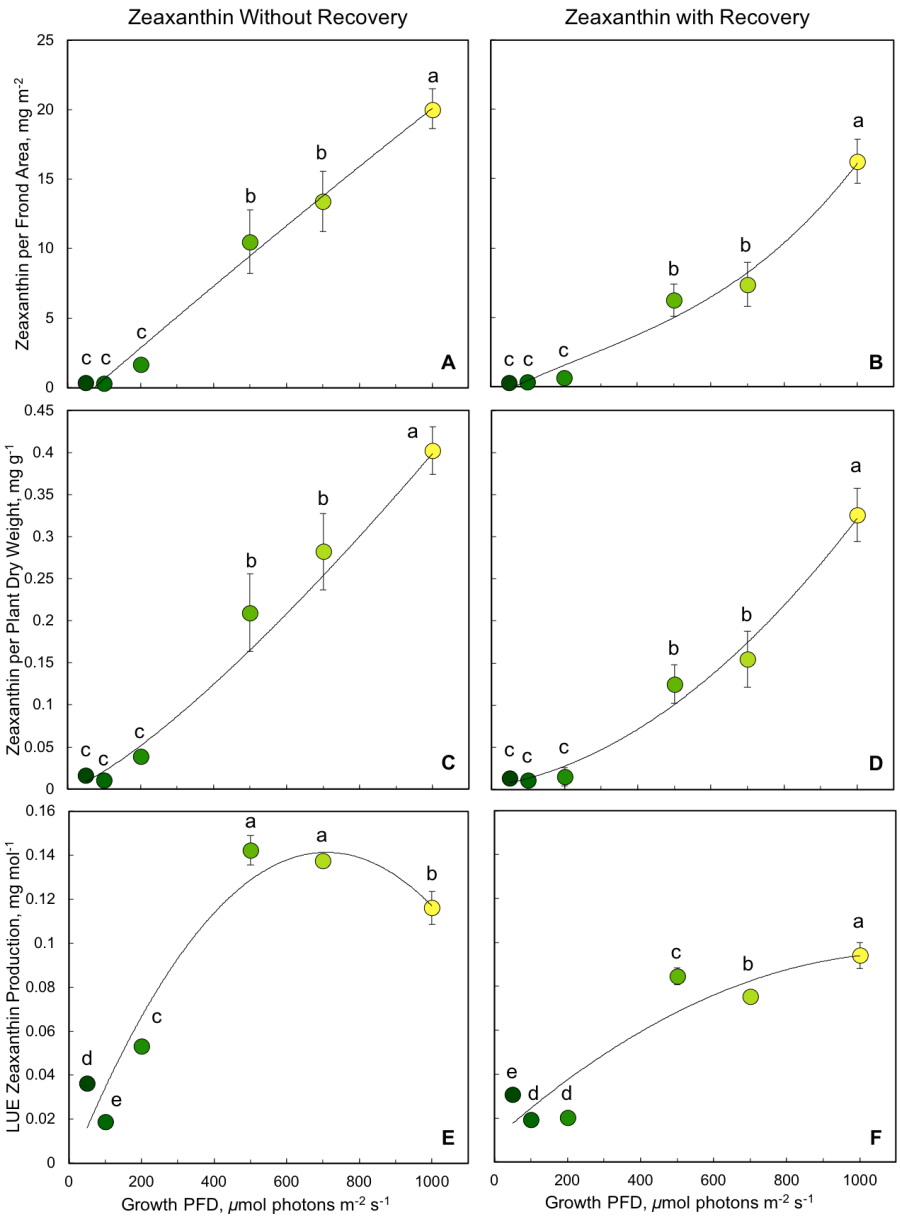

Figure 14. Zeaxanthin production per area (A) without recovery and (B) with a 30-minute recovery period in low light at room temperature; zeaxanthin production per dry biomass (C) without recovery and (D) with recovery, as well as light-use efficiency of zeaxanthin production (E) without recovery and (F) with recovery. Data on zeaxanthin levels in µmol per frond area are from Stewart et al. (2020). Lower-case letters signify significant differences at $p < 0.05$.

5.4 Protein Production

I conducted preliminary testing to determine the protein content of duckweed grown under several light intensities by determining protein content per area under the growth PFDs of 50, 200, and 1000 μmol photons m^{-2} s^{-1}, which showed that duckweed's protein content remained high, and

possibly increased, over a range of growth PFDs (Fig. 15). The fronds analyzed for protein content were grown according to a slightly different protocol than that described in section 4.1 of this thesis, but experiments using the standard conditions are currently underway toward preparation of a manuscript for publication.

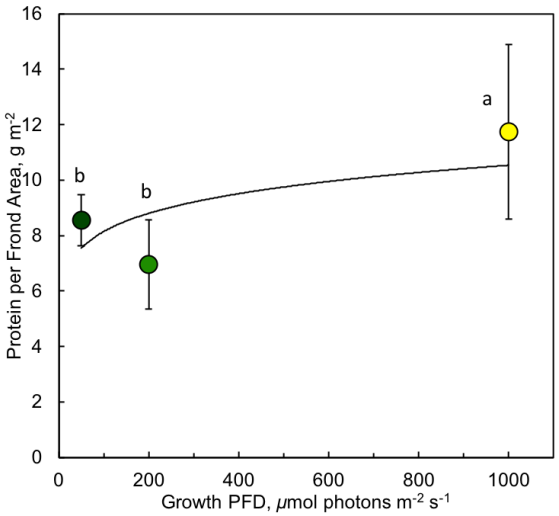

Figure 15. Production of protein per frond area by duckweed fronds grown under three growth PFDs. Lower-case letters signify significant differences at $p < 0.05$.

6. DISCUSSION AND CONCLUSIONS

6.1 Micronutrient Production in Response to Growth PFD

Plants, especially the duckweed species, adjust to different levels of resources provided by diverse growth environments. This response can be seen as a sign of resilience – achieved by a great degree of flexibility. Production of zeaxanthin, lutein, *β*-carotene and tocopherol varied based on the level of protection required in different growth environments. As described in the background section, zeaxanthin production is highest under intense light. This is because zeaxanthin plays a crucial role in dissipating excess energy that a plant might absorb as heat (Demmig-Adams, 1990). Excess light is defined as absorbed light that cannot be fully used in photochemistry and plant growth; this excess excitation energy can lead to ROS formation unless the excess is safely removed, or dissipated, by zeaxanthin (Adams & Demmig-Adams, 1992). When the plant is no longer exposed to intense light, zeaxanthin is quickly removed via conversion back to its precursor (Demmig-Adams & Adams, 1996). In contrast, neither lutein nor *β*-carotene are competitors of photochemistry in plants that should be absent under low growth PFDs; they

instead bind to certain chlorophyll-binding complexes that are less prevalent under high growth PFDs (Demmig-Adams & Adams, 2002; Stewart et al., 2020). Tocopherol was highest under low light and dropped as light intensity increased, but not to the same extent as lutein or β-carotene. This pattern is consistent with the multiple protective roles of tocopherol (Demmig-Adams et al., 2014), including its role in the protection of photosynthesis under low growth PFDs (Niewiadomska et al., 2018).

6.2 Co-Optimization and Remaining Questions

This thesis focused on the production of protein, zeaxanthin, lutein, β-carotene and tocopherol in duckweed samples grown under different light intensities. Duckweed itself grew well over a range of continuous light intensities, which shows its robustness in response to the growth environment. This appears to be achieved by a high degree of flexibility in adjustment of functional components of the plant. In terms of macro- and micronutrient content, the findings of this thesis demonstrate that use of different reference bases leads to slightly different conclusions about what might be considered the ideal growth condition for nutrient production. Overall, the effect of growth PFD on nutrient production was relatively similar for lutein, β-carotene, and tocopherol, while zeaxanthin, as expected, followed an opposite trend. Relative to plant area (with production being rather similar across the range of growth light intensities), moderate light intensities between 100 and 200 μmol photons m^{-2} s^{-1} yielded maximal production of lutein, β-carotene, and tocopherol. Relative to plant dry biomass (with dry biomass production per area increasing with growth light intensity), lutein, β-carotene, and tocopherol production were maximal at an even lower light intensity of 50 μmol photons m^{-2} s^{-1}. Conversely, on both area and dry weight bases, zeaxanthin production (both with and without a recovery period) was negligible under low growth PFDs and kept increasing up to the highest PFD of 1000 μmol photons m^{-2} s^{-1}. An additional reference basis that may be relevant for human spaceflight, and the issue of resource use, is LUE. LUE may provide an important reference to evaluate when considering duckweed for spaceflight applications since the amount of resources available will be highly limited in space and must be used efficiently. LUE was maximal for lutein, β-carotene, and tocopherol at the lowest light intensity of 50 μmol photons m^{-2} s^{-1} due to the fact that the concentration of these micronutrients did not double when light intensity (i.e., investment of light energy) doubled. LUE of zeaxanthin was highest at the growth PFDs of 1000 μmol photons m^{-2} s^{-1} and 500 μmol photons

m^{-2} s^{-1} for with and without recovery periods, respectively, due to the fact that it took a lot of light (energy investment) to produce any zeaxanthin. Zeaxanthin's high rate of production under high light only is consistent with its importance as a photoprotector that removes absorbed light energy when it is excessive. Thus, while optimal conditions for nutrient production differed across the references bases, production of zeaxanthin clearly followed a different trend than that of the other micronutrients. One additional reference basis that should be considered in future research is production per fresh weight, as this is most relevant for the way people actually consume (fresh) food.

Since zeaxanthin levels were high only under very high light, while the other micronutrients peaked under low-to-moderate light, a compromise must be made between production of zeaxanthin and production of biomass and other micronutrients. Co-optimization is complex and should place emphasis on maximizing the concentrations of the most physiologically beneficial micronutrients. Zeaxanthin, lutein, β-carotene, and tocopherol are all crucial to human health, especially in the unique environment of space, but cannot be produced by the body. Zeaxanthin, in particular, is critical to eye health as well as all body functions and, despite their structural similarities, cannot be replaced by lutein (Landrum & Bone, 2001). Zeaxanthin is preferentially accumulated over lutein in the retina of the eye where it plays a crucial role in maintenance of vision (Demmig-Adams & Adams, 2013). Additionally, unlike the other micronutrients that are more readily available in foods, zeaxanthin is very difficult to obtain in the diet (Demmig-Adams & Adams, 2013). Given zeaxanthin's crucial role in maintaining eye health, which is at great risk during spaceflight, as well as the difficulty in obtaining zeaxanthin from the diet, it will be important to maximize duckweed's production of zeaxanthin in space. If astronauts can harvest and freeze the plant immediately, maximal zeaxanthin could be produced with the least energy input under 500 μmol photons m^{-2} s^{-1} (Fig. 14E). On the other hand, if it takes 30 minutes to process and freeze the plant after removal from the growth light source, the plant would need to be grown at 1000 μmol photons m^{-2} s^{-1} to reach the relative highest amount of zeaxanthin per energy input (Fig. 14F). The results presented in this thesis suggest use of one of the first two growth regimes listed below. The third possibility should be addressed by future research on further co-optimizing production of zeaxanthin, lutein, β-carotene, and tocopherol:

- Grow duckweed under an intermediate growth PFD. This will produce reasonably high levels of lutein, β-carotene, and tocopherol. If duckweed is frozen immediately after harvest, zeaxanthin production will be reasonably high as well.
- Growth duckweed under two parallel conditions. One tray of duckweed could be grown under a low growth light PFD to maximize lutein, β-carotene, and tocopherol production. Another tray could be grown under a high growth light PFD to increase zeaxanthin production.
- (To be addressed in future research) Grow duckweed under low growth PFDs with short daily pulses of intense light. This has been shown by our group to increase zeaxanthin concentration while maintaining biomass production in another plant model (Cohu et al., 2014).

It is likely that the only animal protein astronauts will consume on future long-duration missions will be from stored foods since the spacecraft will not be able to host livestock rearing. Since storage space is limited in space, it is important that the renewable, plant-based food source developed for these missions be high in protein. Duckweed's high protein content per area was confirmed by this thesis. In the future, further protein analysis could be conducted to determine protein production across a range of light intensities, which should allow for a better understanding of duckweed's optimal growth conditions for maximal nutritional output. Preliminary protein data presented in this thesis were obtained from duckweed fronds that grew in more densely-packed colonies than those used to obtain data regarding the micronutrients. Testing is currently underway to determine what effect, if any, colony density might have on protein concentration. In addition to light conditions, other environmental factors, including colony density and its impact on nutrient availability, should be considered in the co-optimization process.

As discussed in section 3.5.5, duckweed is also rich in anti-inflammatory omega-3 fatty acids (Appenroth et al., 2017). Since a low ratio of omega-6 to omega-3 fatty acids will be crucial for astronaut health on long-duration missions, future research should also investigate duckweed's production of omega-3 fatty acids across a range of growth light intensities to determine if production is affected by the growth environment and, if so, how.

Furthermore, future research should be conducted to examine the phenolics content of duckweed. The antioxidants examined in this thesis were all water-insoluble, but duckweed also

contains water-soluble phenolic compounds that further support the micronutrients characterized in this thesis. Phenolics play a role in reducing ROS generated by the spaceflight environment (Dangles, 2012). Specifically, phenolics are antioxidants involved in recycling tocopherol (Dangles, 2012) and xanthophylls, including zeaxanthin (Skibsted, 2018; see Fig. 1). Quantifying the phenolics content of duckweed under a range of light intensities will provide further insight into the plant's ability to combat health problems, including ocular effects and system-wide inflammation induced by spaceflight.

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