Discovery of a new species of Heterococcus and analysis of its lifecycle, genome, and lipid production

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Dedication

This dissertation is dedicated to my parents, Jerry Nelson and Andrea Hemphill.

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Chapter 1: “Thesis in context”

Better living through Biology

Increasing population pressure and decreasing reserves of petroleum have provided incentive to discover new sustainable sources of food and fuel. Much attention has been placed on the potential of algae for nutritional purposes and as a feedstock for fuel production (Becker 2007, Simopoulos 1999). The National Renewable Energy Laboratory (NREL) was commissioned to look into this potential in 1978 with the Aquatic Species Program (ASP). Viewed in the context of production cost of algal biofuels vs. cheaper petroleum (at the time), however, the program was discontinued in 1996 due to concerns over production costs. More than 3000 species of algae had been obtained over the duration of the ASP. However, less than 150 species still remain due to limited funding for strain maintenance (Madrigal 2009).

Now, as the reality of the world’s limited supply of oil becomes clearer, laboratories and corporations around the globe are revisiting the potential of algae as a fuel source. However, some already economically viable companies know that fuel is just one use for algae. Any company that is in the business of making food products, pigments, or cosmetics from algae is already able to make a good return from their investment. For example, the market for carotenoids, of which algae is the primary producer, was at 1.2 billion USD in 2010 (BCC Research, FOD025D).

Given the potential of algal systems to supply products of value there should be tremendous pressure to inventory the algal species available in nature to identify those that can produce useful products. However, it is currently unknown by experts in the field how many species exist in nature that are yet to be discovered. Even an intelligent guess is difficult to make, given the limited environmental sampling that has been performed. More extensive surveys of algal species and their products are necessary to elucidate the actual number of extant algal species and their potentially useful products.

If we do not know about so many undiscovered species of algae we cannot find the best strain of algae for any given commercial application. Therefore, strain discovery should be a high priority for future phycology research. Prospecting for new useful species is a relatively untouched area of research, although great progress was made and lost with the ASP.

Life near the freezing point

An abundance of life exists even in extreme temperatures. Heat-tolerant microorganisms are known as thermophiles while cold-tolerant microorganisms are known as psychrophiles or cryophiles. Psychrophiles have an optimal growth temperature at about 15°C or lower, a maximal temperature for growth at about 20°C and a minimal temperature for growth at 0°C or lower (Moyer *et al.* 2007). Psychrophiles have been known to grow at temperatures ranging from -20°C to 10°C (D'Amico *et al.* 2006). Some cold-tolerant organisms, depending on their

optimal growth temperature, could also be called either psychrotolerants or psychrotrophs (Morita, 1975). Psychrotrophs can grow at low temperatures but have optimal and maximal growth temperatures above 15 and 20°C, respectively (Moyer *et a.l* 2007).

Psychrophiles are very relevant to applications-based science and biotechnology because of the unique properties of their cellular components including certain enzymes, antifreeze proteins and exopolysaccharides (Chavecchioli *et al.* 2011, Margesin and Feller 2010, Nicolaus *et al.* 2010, Paredes *et al.* 2011, Piette *et al.* 2011, Wilson and Walker 2010, Xiao 2010). As an example the cellulases from psychrophiles find applications in molecular biology, the food industry, and environmental bioremediation.

The metabolism of psychrophiles is much different from that of mesophiles. Psychrophiles raise levels of intracellular adenosine 5'-triphosphate (ATP) as temperatures decline so as to keep available energy high in lieu of decreased molecular motion and thermal energy (Feller 2007). In addition, psychrophiles tend to have less adenosine 5'-monophosphate (AMP) degradation enzymes and more AMP synthetic enzymes than mesophiles (Parry and Shain 2011). Keeping AMP levels high for ATP synthesis is an excellent example of how psychrophiles deal with lower temperatures. In fact, Perry and Shain (2011) found that manipulating AMP metabolism in *E. coli* led to its growing up to ~70% faster at low temperatures.

In addition to metabolism, a host of other cellular processes have undergone adaptation by psychrophiles to function at low temperatures. The most critically important requirement for life at low temperatures for psychrophiles is the maintenance of functional cellular membranes at exceptionally low temperatures (Thomas and Diekman 2002). Because of the deleterious effects of low temperature on membrane fluidity and enzyme catalysis, the composition of membranes and enzymes must be selected to have favorable characteristics to function at extrememly low temperatures (Goodchild *et al*, 2004, Mykytczuk *et al.* 2010, Ratkowsky *et al*, 2005, Siddiqui and Cavicchioli 2006). Enzymes and chromatin structure must also be folded properly, which mesophillic organisms cannot do at low temperatures, hence a need for psychrophillic adaptations to these components as well (Siddiqui and Cavicchioli 2006).

Algae in cold climates

Among the myriad species of organisms adapted to cold climates, algae are primary producers of nutrients essential for the other organisms in the ecosystems they inhabit (Arrigo *et al.* 1997, Bluhm *et al.* 2008, Cardinale *et al.* 2011). Algae bring organic carbon into cold ecosystems that can then be used by bacteria, fungi, and higher organisms, although cold ecosystems in general sequester less carbon than most other ecosystems on Earth (Callaghan *et al.* 2004). The variation of productivity in cold ecosystems is due to the duration/depth of winter snow cover, degree of protection from winter wind

damage, variation in soil moisture, soil thaw, and soil temperature (Callaghan *et al.* 2004). Local variation in these factors can be nearly as great as that across a wide range of latitudes (Shulze *et al.* 2001). Environmental suitability may be why the same genus of algae (*Heterococcus)* can be found in Antarctica and Colorado: the climate on the top of a mountain is very similar to some areas in Antarctica. Thus understanding the ecophysiology of arctic algae may help in understanding the ecophysiology of alpine algae.

Unfortunately, due to global warming, major shifts in arctic environments have been occurring that may have resulted in massive losses of algal species (Smol *et al.* 2004). However, vast numbers of algal species still populate northern and southern arctic areas and these areas are the most popular destinations for researchers interested in cold-adapted algae. Algae species in the northern Arctic include *Chlamydomonas nivalis*, Scotiellopsis sp., *Klebsormidium flaccidum,* Zygnema *sp., Meridion circulare, Tabellaria fenestrata* and Fragilaria *sp.* (Kim *et al.* 2008). Algae species in the southern arctic (Antarctica) include species such as *Pheocystis antarctica* and *Stichococcus bacillarus* (Chen *et al.* 2012) as well has a number of species of *Heterococcus.* The species of algae found in cold climates span many diverse taxa. Green algae (Gorton *et al.* 2010), yellow-green algae (Rybalka *et al.* 2009), red algae (Lohrmann *et al.* 2004), golden algae and picobiliphytes (Weber *et al.* 2012) have all been recovered from cold environments and studied to various extents.

Algae that live in cold climates have different responses to environmental changes and different fitness levels depending on the environmental changes involved. Cyanobacteria have different responses to cold, wind, sun, etc. than do eukaryotic algae. Cyanobacteria more readily respond to strong fluctuation of solar radiation levels, desiccation cycles, salinity and freeze-thaw cycles by modulating expression of adaptive genes to mitigate these stresses (Callaghan *et al.* 2004). Overall, prokaryotic psychrophiles are more resistant to harsh conditions. On the other hand, eukaryotic algae cannot respond as readily to extreme environmental changes but do have higher rates of photosynthesis (Callaghan *et al.* 2004). Thus given more extreme cold conditions the prokaryotic organisms would out-compete the eukaryotic algae and in more mild conditions the eukaryotic algae would out-compete the prokaryotes.

Typical locations of cold-adapted algae are in cold ocean water, snow fields in places such as Antarctica and Siberia, within pack ice, and atop mountains. Algae in arctic deserts can be found under rocks and in crevices (Thomas 2005). Algae in pack ice can even be found in freeboard layers (layers at sea-level covered by snow) but are more likely to be found high in floes where sunlight is more abundant (Brierly and Thomas 2002). Algal growth in these areas in pack ice can be so much as to contribute to ice melting from increased absorption of sunlight (Ackley and Sullivan 1994).

Algae can also be found in glaciers, which may explain the vast geographical spread of some families of algae. Glacial algae tend to be found in

cryoconite holes, medial moraines, and supraglacial kames (Stibal *et al.* 2006). In glaciers, the highest density of algae is found in cryoconite holes (holes made from absorption of sunlight by dark sediment) because these locations receive nutrient-rich sediments blown by wind and washed by rains (Stibal *et al.* 2006). Likewise, any area where nutrients accumulate is likely to support greater populations of algae than exposed surfaces. For example, there is likely to be much more algal growth in the trough of a mountain side than the peak or ridges. Water containing sediment and avian waste is more likely to flow through these trough areas and bring nutrients to algae supporting growth. Increased flooding in snowy upper layers of glaciers has been shown to increase primary production by algae (Arrigo *et al.* 1997). However flooding does not imply homogenous distributions of algal species in glaciers or pack ice. Large-scale secondary pores such as cryoconite holes may contain highly variant ecosystems unique to each hole. Such uniqe individual sub-ecosystems create a high degree of variation of algae species in geographically similar locations in pack ice (Eicken *et al.* 1991).

Many cold-adapted algae are studied as symbiotes in association fungi and perhaps one or more species of bacteria, commonly known as lichens. Lichens are incredibly cold-resistant (Ascaso and Wierzchos 2002, Cornellisson *et al.* 2007. In some colder climates lichens provide the dominant food source for some primates (Black-and-white or Yunnan snub-nosed monkeys) specifically because of their abundance at cold temperatures (Grueter *et al.* 2009). Many

lichens have been shown to benefit from being frozen over (Bjerke 2009). Ice encapsulization has been shown to increase productivity and photosynthesis compared to non-encapsulated lichens in the same environment (Bjerke 2009).

Alpine algae have not been extensively studied in most alpine areas of the world and are not well characterized (Novis *et al.* 2011). A few eukaryotic isolates have been studied in New Zealand, however the small number of isolates found are likely only a small percentage of the alpine algae that exist throughout the world (Novis 2002, Novis *et al.* 2008).

In addition to cold-tolerance, alpine algae must also be resistant to the high levels of solar irradiation found above the tree line. Solar radiation has been shown to affect growth, photosynthesis, nitrogen incorporation and enzyme activity in photosynthetic microbes (Hader 2000). Alpine algae must have in place mechanisms to repair or prevent double-strand breaks from higher gamma ray levels and thymine dimers caused by higher-than-normal levels of UV radiation (Marquis *et al.* 2009, Vlcek *et al.* 2008). A simple way to deal with this problem among alpine life is simply to increase pigmentation or accumulate specific types of protective pigments including certain carotenoids and xanthins (Gorton *et al.* 2001, Holzinger *et al.* 1993, Takaichi 2011). Whatever adaptation acquired, some algae seem to have become impervious to the normally damaging effects of solar radiation. For example, the arctic freshwater alga *Zygnema sp.* is completely insensitive to experimental UV radiation reaching up to 1300 μmol photons m^{-2} s^{-1} (Holzinger *et al.* 2009).

Oil production in psychrophiles

In order to keep biological membranes from becoming too rigid in cold temperatures, psychrophiles must maintain a membrane composed of fatty acids with high degree of unsaturation (Quinn 1988). In general the colder the normal environmental temperature for an organism, the more double bonds it will have in its membrane fatty acids and the longer these fatty acids will be. Fatty acids with chain lengths of 16-18 C and 0-3 double bonds comprise the majority of membranes in mesophiles while fatty acids with chain lengths of >20 C and more than 3 double bonds are more abundant in psychrophile membranes. This correlation is directly due to the thermodynamics of membrane structure and hydrocarbon plasticity.

In addition, psychrophiles tend to store more oil than mesophiles to compensate for the long and dark winters often found in cold climates (Logue *et al.* 2000). During the winter carbon stores are depleted. Photosynthesis is limited as day length shortens and the light intensity declines as the sun lowers in the sky. One strategy to deal with seasonal changes is to maintain an annual period of oil accumulation and a period of oil catabolism (Blumental *et al.* 2011).

Yellow-green algae

The yellow-green algae, or Xanthophyceae, are a group of algae traditionally containing the orders Botrydiales, Mischococcales, Tribonematales, and Vaucheriales. Yellow-greeen algae are mostly fresh-water algae although some species inhabit marine environments (Stace 1991). Many species inhabit soil or rocky environments (Darling *et al.* 1987). Yellow-green algae are named for their light yellow green colour. The lightness of the colour is due to the absence of the brown pigment fucoxanthin which gives most other photosynthetic organisms darker hues of green (Stace 1991). Yellow-green algae do not accumulate starch as most other heterokonts do; instead they accumulate the storage polysaccharide crysolaminarin (Stace 1991).

Classification of yellow-green algae is currently incomplete even among known species (Adl *et al.* 2005, Negrisolo *et al.* 2004). Many groupings are polyphyletic, sometimes even among genus distinctions and it has been suggested that at most two orders should be maintained within the yellow-green algae (Adl *et al.* 2005, Maistro *et al.* 2007). Current proposed orders are: Botrydiales, Mischococcales, Tribonematales, and Vaucheriales, with Tribonematales and Botrydiales considered to be polypheletic and Mischococcales considered to be paraphyletic from recent ultrastructural and phylogenetic studies (Adl *et al.* 2005). Relatives of the yellow-green algae include Chrysophyceae, Eustigmatophyceae, Phaeophyceae, and

Synurophycea. The most closely related group to the yellow-green algae are brown algae, or Pheophyceae, including macroalgae such as *Ectocarpus siliculosus* and *Macrocystis pyrofera* (Adl *et al.* 2005). Presented in this thesis is a newly discovered species of algae found in the Colorado Rockies, US. This species of oil-accumulating algae was identified by molecular data and morphology (chapter 2) to belong to the yellow-green algae and more specifically the genus *Heteroccocus.*

Early characterization of yellow-green algae was limited to only very basic life-cycle analysis and culture growth studies (Miller and Fogg 1958). *Modus subterraneus* was used as a representative yellow-green alga for many of these studies. *M. subterraneus* was found to grow best on a media designed for blue-green cyanobacteria, Chu M 10 (Chu 1942). No exogenous organic compounds were found to support heterotrophic growth of yellow green algae in the dark. The addition of easily oxidizable organic compounds like sugars provided no boost to the growth of *M. subterraneus* (Miller and Fogg 1958), *Visheria stellate* or *Pleurochloris communtata (Casselton 1965)*. However, the addition of peptone preparations like “Bacto-peptone” and “Bacto-tryptone” (Difco) resulted in higher growth rates and higher final cell mass of when added to aerated cultures of *M. subterraneus* in Chu M 10 medium (Miller and Fogg 1958). Exogenous carbon compounds are used by other yellow-green algae. Glucose is used as a carbon source by *Chloridella neglecta, Botrydiopsis arhiza,*

Chlorocloster solani, Bumilleriopsis peterseniana and *Heterococcus caespitosus* (Casselton 1965).

Heterococcus is rarely found in nature. Even when *Heterococcus* samples can be obtained, they are only found as single cells or fragments of filaments and are very difficult to culture (Pascher 1939). The distribution of *Heterococcus* is restricted to alpine and polar regions, providing evidence that this genus is psychrophilic (Darling and Friedmann *et al.* 1987). Although the genus may be classified as psychrophilic, this newly-discovered strain, which we have named, *H. coloradii,* grows robustly at 22ºC. The discovered strain of *Heterococcus* can be characterized as psychrotolerant. Other *Heterococcus* species may be characterized as psychrotolerant algae as well even though no strains of *Heterococcus* are found outside of alpine or polar regions. The reason for the restricted biogeography of *Heterococcus* is probably that *Heterococcus* has no competitive edge at elevated temperatures. Mesophillic organisms have a steep growth advantage over strains of *Heterococcus* and would out-compete *Heterococcus* in any conceivable environment with moderate temperatures. Nevertheless, *Heterococcus* has previously been found to grow on four different continents: Europe, North America, Antarctica and Zealandia. The previous North American species of *Heterococcus, H. canadensis,* was the only species reported in North America until the discovery of *H. coloradii.*

A total of 62 species of *Heterococcus* are listed in Algaebase, a comprehensive website/data bank that compiles information regarding the identification, distribution, and relevant literature of land, marine, and freshwater algae (http://www.algaebase.org/). Currently the algae culture collection at UTEX (University of Texas at Austin) holds 9 species of Heterococcus although none of the species are from snow or North America.

Algae in industry

The goal of the ASP was to identify species of algae that could produce fuel precursors in a marine environment. Actually, algae have been used for hundreds of years to make a variety of products useful to humans (Sanghvi *et al.* 2011). Now the algae industry uses algae to make products ranging from foodstuffs to medicine to cosmetics (Becker 2007, Christaki *et al.* 2011, Imhoff *et al.* 2011, and Sanghvi *et al.* 2010). Among the metabolites of algae used for industrial purposes are: fatty acids, halogenated compounds, steroids, lectins, carotenoids, polysaccharides, amino acids, polyketides and toxins (Cardozo *et al.* 2007). Over 15,000 novel compounds have been discovered from various species of algae so far (Cardozo *et al.* 2007).

Algae are desirable to use as feedstock for farm animals because of their nutrient content. Algae grown as a feedstock for animals is high in nutritional fatty acids, vitamins, fiber, minerals, carbohydrates and proteins (Becker 2007, Cardozo *et al.* 2007, Nakagawa 1997, Simopoulos 1999). Animals that benefit

from supplementation include cattle, pigs, fowl and fish (Becker 2007). Since animals fed and supplemented with algae are for human consumption it is ultimately humans who receive the benefits of supplementing animal feed with algae.

The nutraceutical industry has seen dramatically increased demand for algae as nutritional supplements, although many countries have been supplementing their diets with algae for centuries (Kahn *et al.* 2010). This is especially true in Asian countries, where marine algae are commonly eaten daily and for special purposes. In Japan it is very common to eat a seaweed salad before consuming sushi wrapped in Nori, or dried seaweed. In Korea, mothers traditionally consume *Miyeok-guk,* a hot and spicy algae soup, during the month after delivering their babies. This soup is commonly believed to supply the necessary nutrients to aid in post-natal recovery.

The anticancer properties of consumed algae have been reviewed extensively (Chakraborty *et al.* 2009, Jiang *et al.* 2011, Liu *et al.* 2011, Pisani *et al.* 2002). *Palmaria palmate, Laminaria setchelli, Macrocystis integrifolia, Nereocystis leukeana, Udotea flabellum, and Udotea conglutinate* are species of algae that have already been shown to inhibit cancer cell proliferation in vitro (Moo-Puc *et al.* 2009, Yuan and Walsh 2006, Yuan *et al.* 2005). In addition to anticancer activity, a variety of algae have been shown to have antiviral and antiobesity activity (Kim *et al.* 2011).

Carotenoid production from algae is commercially important since algae make carotenoids in large quantities and carotenoids can be very expensive. Carotenoids made by algae include the valuable pigments astaxanthin and beta-carotene. Astaxanthin is the most expensive and important pigment made by algae (Margalith 1999). Astaxanthin is widely used as a food colorant and has many uses as a nutraceutical for its free-radical scavenging, immunomodulation and cancer prevention properties. Astaxanthin is made by the freshwater flagellate *Haematococcus pluvialis* (Chlorophyceae) that has red cells due to pigment accumulation. Beta-carotenes can promote vitamin A production and have been shown to prevent cancer, promote ocular health and mitigate some photosensitivy conditions (Mayne 1996). Beta-carotene is made predominantly by *Dunaliella salina*, an orange-colored species that can also synthesize a number of other valuable compounds (Hosseini *et al.* 2009). The global market of beta-carotene, dominated by *D. salina*- produced beta-carotene, is valued at over 261 million USD per year (BCC Research FOD 025D). Obviously the harvest of all of the biological components of an alga used for a commercial biotechnology purpose is highly desirable.

Lipid/biofuel production in algae

The number of literature reports regarding lipid production in algae has greatly accelerated with the recognition of algae as a potential source of biofuels. Algae are being considered as a source for fatty acids, mostly in the form of

triglycerides, to be processed into biodiesel. Biodiesel is the methyl-ester product formed by reacting algal lipids with methanol and sodium or potassium hydroxide. Biodiesel can be used as a drop-in substitute for normal diesel fuels provided functioning conditions are not extreme. For example, biodiesel is not suitable for use at very low temperatures because of its high gelling temperature or in high-performance vehicles because of its low flash point. Nevertheless biodiesel works well as a general purpose fuel and little to no modification is required for optimal performance of diesel engines using biodiesel fuel. For example, undergraduate students at Colorado College routinely use in-house made biodiesel from kitchen oils to power diesel-fueled grounds maintenance machines such as tractors and mowers.

High lipid/hydrocarbon-producing species of algae include *Botryococcus braunii* (Eroglu *et al.* 2011, Ioki *et al.* 2011, MacDougal *et al.* 2011, Niitsu *et al.* 2011, Yonezawa *et al.* 2011), *Thalassiosira psuedonana*, (Jiang *et al. 2012), Nannochlopsis sp.* (Kilian *et al.* 2011, Mohammady 2011, Pal *et al.* 2011, Quinn *et al.* 2012, Simionato *et al.* 2011), *Dunaliella sp.* (Araujo *et al.* 2011, Chen *et al.* 2011, Krohn *et al.* 2011, Rizmani-Yazdi *et al.* 2011, Yang *et al.* 2011), and *Chlamydomonas reinhardtii* (Fan *et al.* 2011, Kropat *et al.* 2011, Nguyen *et al.* 2011, Torri *et al.* 2011, Wang *et al.* 2009, Wang *et al.* 2011) *Duniallа tertiolecta* and *T. psuedonana* gave lipid yields of 20-26% (Jiang *et al.* 2012), *Nannochloropsis* gave lipid yields of 47.5 ±7.1% (Radakovits *et al.* 2012), and *Chlamydomonas* gave lipid yields of 0.9-20% (Ratha *et al.* 2012).

In most algal cells, nitrogen deprivation is a strong signal for lipid production (Araujo *et al.* 2011, Chen *et al.* 2011, Jiang *et al. 2012,* Ratha *et al.* 2012), although high light (Liu *et al.* 2012) and sulfur (Cakmak 2012) deprivation have been used with varying effectiveness. The main strategy used for commercial production of lipids is to grow algae past logarithmic stage to stationary phase then remove all nitrogen from the media to produce the highest yields of lipids (Hannon *et al.* 2012).

Botryococcus is a species of green algae (*Chlorophyceae)* that excretes hydrocarbons into its surrounding media. It has been shown to accumulate up to 55% hydrocarbon fuel precursors by dry weight (Ruangsomboon 2011). One drawback of using *Botryococcus* for biofuel production is its slow growth rate, as the cells average about one doubling per three days (Ashokkumar *et al.* 2011).

Nannochloropsis is a small yellow-green alga that is widely used as a feedstock for algal biofuel production. Light intensity was not found to be a strong signal for lipid production in *Nannochloropsis* (Simionato *et al* 2011), however nitrogen deficiency was found to induce lipid accumulation (Pal *et al.* 2011). An extremely useful advantage to *Nannochloropsis* is that it has efficient homologous recombination which is highly advantageous for genetic engineering projects (Kilian *et al.* 2011).

Dunaliella salina and *Dunaliella tertiolecta* are the most common strains of *Dunaliella* used for biofuel production. Species of *Dunaliella* are commonly found in salt-water environments, such as the Dead Sea in Israel or the salt flats of

Utah that may reach up to 5.5 M NaCl (Chen *et al.* 2009). In addition to biofuels, *Dunaliella* is commonly used for the production of beta-carotene and the production of exopolysaccharides, poly-beta-hydroxyalkanoate bioplastics, and biofuel are being investigated (Oren 2010).

Chlamydomonas reinhardtii is a green alga that is a model algal system for studying cilia/flagella function (Silflow and Lefebvre 2001), as well as many other biological processes including circadian clock function (Matsuo 2011), and DNA repair in phototrophic eukaryotes (Vlcek *et al.* 2008). Lipid production in *C. reinhardtii* is triggered by nitrogen starvation (Wang *et al.* 2009) or iron or zinc deficiency (Kropat *et al.* 2011). Starchless mutants defective in an ADP-glucose pyrophosphorylase hyper-accumulate triglycerides (Li *et al.* 2010). This hyper-accumulation occurs presumably because energy that would normally be stored in starch is instead shunted to the triglyceride production pathways. Wang *et al.* (2009) documented the extensive lipid accumulation in a starchless *C. reinhardtii* mutant deprived of nitrogen. Other efforts to optimize *C. reinhardtii* for more efficient lipid production include optimizing photosynthetic efficiency. By downregulating the amount of light-harvesting proteins, a 30% increase in photosynthetic efficiency was obtained in *C. reinhardtii* (Mussgnug *et al.* 2007).

Studies on biodiesel production from algae vary in predicted yields from 80 tonnes per hectare to 125 tonnes per hectare per year (Rhodes 2009). Using the most ideal estimate, 200,000 hectares of land could produce one quad, or 7.5 billion gallons, of biodiesel annually, or 205 million gallons daily. Since the U.S.

uses uses an average of 378 million gallons of transportation fuel a day (U.S. E.I.A. 2009), 400,000 total hectares of land for growing algae would be more than enough to supply all of the transportation fuel the U.S. requires. There is no doubt biofuel production from algae is physically possible, however the problem is its market feasibility.

Estimates for the cost of biofuel production using conservative figures from the Molina Grima 2003 and Department of Energy reports, cost per gallon estimates of $1300 and $115 were made for closed and open bioreactor systems for photoautotrophically grown algae (Gao *et al.* 2012). These estimates used an assumed 10% lipid yield and $0.47/kg CO_2 which are quite conservative since many algae are known to produce lipids at around 50% of their total mass (Rhodes 2009) However, using the generous assumptions of 30% lipid yield and $0.2/kg CO_2, prices were estimated to be under $1.94/gallon (Gao *et al.* 2012).

In order for lipid/biodiesel production to be sustainable and economically viable in algae, production will need to use photoautotrophically grown cells. Although a number of companies are using feedstocks such as acetate or sugars, these carbon compounds can be expensive and are often non-renewable. For example, Solazyme, a biofuel-from-algae company that is now traded publically, uses a patented process to create biofuels that utilizes stressed algae in the dark fed with massive amounts of sugar. A recent estimate puts 75% of the cost of algal biofuels on supplying acetate alone (where acetate is used, Menetrez 2012). Although some scientists believe that carbon feedstocks

are necessary to produce high yields of algal biomass (>50 g/L) in liquid media (Algal Biomass Organization, personal correspondence), we have yet to discover the correct organism(s) for photoautotrophic production of biofuels.

Using photoautotrophic organisms for the production of biofuels is also important if we are to remove CO_2 from the atmosphere using algal growth. In addition to its dangerous effects on global weather patterns (Fiore *et al.* 2012, Tollefson 2012), global warming, triggered at least in part by CO2 accumulation, has been reported to have significant impacts on human health (Epstein 2000). Use of algae by humans to lower atmospheric CO_2 could offset the rise in global warming. Technologies such as the high-rate pond (HRP) method of growing algae have potential to remove CO_2 from the atmosphere (Tsai *et al.* 2011) Researchers are working to develop bioreactors suitable for carbon sequestration (Kumar *et al.* 2011). Such bioreactors can connect to flue gas outlets from coal-fired power plants and significantly reduce toxic emissions such as CO_2, $SO_{(x)}$, and $NO_{(x)}$ that would otherwise promote global warming.

Another potential problem with algae biofuel production is the cost and shortage of fertilizers. Although Gao *et al.* 2012 cited CO_2 as the most cost-prohibitive nutrient, other researchers believe that nitrogen and phosphorous are more problematic for large-scale algae biofuel production. Lissa Morgenthaler-Jones, the CEO of the algae company LiveFuels (CA), was quoted as saying “The truth is, neither [Venter nor Keasling] will succeed in replacing petroleum for many reasons, including the fact that [genetically modified organisms] are not as

robust as wild species. But what may be the biggest reason was covered by *Foreign Policy* months ago - the looming phosphate shortage." However, this problem may be solved by the fact that algae are efficient scavengers of phosphate from waste water, and waste water treatment facilities are looking for ways to remove phosphate from waste water.

Algae need nitrogen, phosphorous and carbon to grow and supplying the former two nutrients is so expensive so as to make fuel production cost-prohibitive (U.S. DoE, 2010). Other nutrients required are potassium, iron, manganese, sulfur, zinc, copper, cobalt, silica and calcium. All of the necessary nutrients can be supplied by using wastewater (Knud-Hansen *et al.* 1998).

Biodiesel production from algae is unlikely to be economically feasible in the near future without the use of wastewater. Estimates of algal biomass production using municipal waste per person were between 13 and 59 g per day (Boelee *et al.* 2012, Young Algeneers Symposium). However, even assuming high lipid yields (%50), this would at most be 30g of lipids for biodiesel per day per person. 30g is hardly a fraction of the average commuter's daily fuel expenditure of at least 1-2 gallons. Even so, growing algae from wastewater has the added benefit of removing nitrogen and phosphorous which would otherwise cost money. A group from Spain was able to achieve 95% removal of NH_4 from wastewater effluent of 35 mg NH_4/L in 6-10 days (Ferrer *et al.* 2012, Young Algeneers Symposium).

The United States currently spends $46 billion annually on wastewater treatment (U.S. CBO, 2002). The total reported wastewater flow in the U.S. is 32 billion gallons per day (U.S. EPA, 2008, CleanWatersheds Needs Survey). Assuming a 10% lipid yield (Woertz *et al.* 2009), a biodiesel density of 0.80 kg l^{-1} (Vijayaraghavan and Hemanathan, 2009) and 9 months per year operation, an average biodiesel production of 1.7 million gallons per day can be produced from wastewater-treated algae in the U.S. (Christenson and Sims, 2011).

The U.S. uses an average of 378 million gallons of transportation fuel a day (U.S. E.I.A. 2009), so biodiesel produced from wastewater-grown algae would still only be less than one percent of the total fuel consumed in the U.S. In order for algal biodiesel to replace a larger fraction of our transportation fuels, non-wastewater grown algae production costs will have to be lowered dramatically and lipid production yields will have to increase dramatically. Given the wide range of estimates for photoautotrophic production of biofuel from algae we should not jump to conclusions lightly, but perservere in research attempts that may have yet unknown benefits. Even if biodiesel from algae is not economically feasible in the near future, there is still high potential for the production of specialized products such as nutritional supplements and medicines from algae.

This dissertation describes the discovery and characterization of a new species of algae that may be useful for the production of lipids for biofuels and for long chain polyunsaturated fatty acids for human health. Other implications of

this work include the discovery of putative algal antifreeze proteins which may be useful in crop freeze-tolerance experiments. Because currently unknown species of algae exist in the world, bioprospecting and subsequent characterization of new algal species may prove to be valuable in the future. Instead of trying to engineer the perfect strain of algae for a certain commercial application, a company might utilize a strain with the desired properties already existing in nature.

Chapter 2: New lipid-producing, cold-tolerant species of *Heterococcus* isolated from the Rocky Mountains of Colorado[1]

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Condensed: Novel species of *Heterococcus*

A new species of *Xanthophyceae*, *Heterococcus coloradii*, was discovered among snow fields in the Rocky Mountains. The environmental sample containing *H. coloradii* also contained three other species of algae and several species of fungi and bacteria, all of which were cultured in the laboratory using a minimal salts media. Axenic cultures of *H. coloradii* were isolated, and their cellular morphology, growth, and accumulation of lipids were characterized. *H. coloradii* was found to grow at temperatures approaching freezing and to accumulate large intracellular stores of lipids. Of particular interest was the accumulation of several long-chain polyunsaturated fatty acids known to be important for human nutrition. Algae that accumulate lipids in this manner have potential uses as sources of biofuels and poly-unsaturated fatty acids for human nutrition.

Key index words: algae, oil, lipids, algal oil, cold-tolerant, desiccation-tolerant

INTRODUCTION

We describe the discovery of a novel species within the yellow-green algae recovered from the Rocky Mountains of Colorado in the United States. The presented isolate was determined to belong to the genus *Heterococcus* and was selected for study because of its high degree of lipid accumulation. The genus *Heterococcus* was established by Chodat in 1908 and encompasses more

than 48 species to date, many of which have been found in Antarctica (Rybalka *et al.* 2009). Species of *Heterococcus* have been recovered from Europe and Antarctica although no reports describe species of this genus from North America. Isolates are commonly found in cold terrestrial ecosystems (Rybalka *et al.* 2009), although *H. coloradii,* the new species described in this report, was cultured directly from an alpine snow sample.

MATERIALS AND METHODS

Culture: Snow was collected in a small water bottle at about 13,000 ft elevation on Peak 10 of Breckonridge, Colorado. Drops of the melted snow were spread on minimal *Chlamydomonas* agar media (1.2% agar, Sager and Granick medium I (SGI), Harris 2010) and incubated under constant light at 4 º C.

Green colonies of varying morphology were transferred to liquid media using sterile toothpicks and after growth at 4º for 1 week the morphology of resulting cells was examined by phase contrast microscopy. Staining with a lipophilic dye, either Nile Red or Bodipy 505/515, showed that one of the species of algae recovered accumulated large amounts of lipids in abundant spherical intracellular droplets of varying sizes. Each colony with a unique morphology was streaked out and re-tested with Nile Red. The original colony of *H. coloradii* was isolated as a round colony with cell chains and tested positive for high levels of intracellular lipid stores via Nile Red staining.

Axenic cultures were obtained by spray-plating a small amount of liquid culture so that individual droplets formed colonies (Harris, 2010). Briefly, an aerosol of cell cultures was created by blowing filtered air over one end of a 25 µl pipet immersed in the culture solution. Agar plates were passed through the resulting aerosol cloud to allow individual cells to bind to the plate and grow, over the next three days, into colonies. The microdroplets were so small that some individual colonies contained only the single species of interest which separated it from contaminating organisms. As a final step, colonies were streaked out on plates containing three antibiotics: streptomycin (1 g/L, Sigma), ampicillin (100 mg/L, Sigma), timentin (1 g/L, Agri-bio). Colonies were picked after 2-3 weeks of growth on the antibiotic plates to create working cultures of *H. coloradii.*

Identification: H. coloradii was initially classified by its extraordinary life cycle. After adequate cell densities were acquired for DNA extraction, a fragment of 18S rDNA was amplified using polymerase chain reaction (PCR) and the sequence of the resulting PCR product was compared with other nucleotide entries using the BLAST program at the National Center for Biomedical information (NCBI) at the National Library of Medicine. A BLAST (Basic Local Alignment Search Tool) was performed against the NCBI nucleotide database with a 322 base pair PCR-amplified fragment from *H. coloradii*. The top 4 BLAST results are shown in Table 1.

Molecular methods**:** DNA from the axenic cultures was isolated through either purification using a kit (Gentra) or by simply lysing the cells to release DNA for PCR using a colony PCR method (Cao *et al.* 2009). 18s rDNA was amplified using PCR. Thermocycling parameters were: 94°C for 5 minutes, then 35 cycles of 1 minute at 95°C, 1 minute at 56°C, and 2 minutes at 72°C. Cycling was followed by a 5 minute extension at 72°C. Pfu Polymerase (Promega) was used along with its buffer to amplify sequence according to the manufacturer's specifications. The final PCR product was sequenced for comparison with NCBI nucleotide entries, as in Table 1. Primers used for amplification of rDNA were: CCTGCCAGTAGTCATACGCT (forward, nt 5-20, Andreoli et al. 1999) and CCCAGAAATCCAACTACGAG (reverse, nt 667-647, Negrisolo et al. 2004).

Cell division and growth: Growth curves were generated for cells grown on different media including Sager and Granick I media (Harris 2010), solid and liquid BG-11, Bristol's media, Bold's basal media (all media recipes can be found at: http://www.sbs.utexas.edu/ utex/media.aspx) and other in-house media formulations (Fig. 6, SG1 measurements made by David Nelson, other measurements made by Sinafik Mengistu). Cells were grown under constant light (10,000 lux) and at either 22 or 4º. Growth was measured as an increase in biomass (dry weight). To measure dry weight, cells were inoculated onto 70 mm Whatman filter paper discs on top of the different agar-based media. Each filter was harvested as one sample, and triplicate samples were taken for each day

measurements were made. Filter paper discs with algae were baked in a vacuum oven at 80°C for 2 hours before weighing to determine dry mass.

Cell counting was performed using a FACSCalibur flow cytometer (FCM) with the CellQuest Pro software. Before each run the FCM was washed with bleach for 10 min. 20 μl of a 2 ml culture was added to 1 ml of FCM running buffer and fed to the FCM with a flow rate of 12 μl/min. Each run was 50 seconds with 3 runs per sample. An FSC amplifier and FL1,FL2 SSC, FL1, FL2, FL3 and FL4 detectors were used. Amplifier/detector settings: FSC: E01v (log), SSC: 415v (log), fl1: 505v (log), fl2: 550v (log), fl3: 650v (log).

Fluorescence microscopy: *H. coloradii* cells were stained with Nile Red in DMSO (Wang *et al.* 2009) for at least 2 hours prior to imaging. Cells were viewed at 1000x and 400x with a Diaplan differential interference contrast/fluorescence microscope filtered for 468 nm light and captured with a Jenoptic digital camera. Scale bars were created from measurements made with a 10 μm Carl Zeiss stage micrometer.

Lipid extraction: Lipids were extracted from *H. coloradii* using methyl *tert*-butyl ether (MTBE) according to a protocol developed by Matyash *et al.* (2008). The authors of this protocol reported more complete yields with MTBE than with traditional hexane extractions (Folch *et al.* 1957). Lipid extractions were done with more than 10 different cultures that had been growing anywhere from 2 to

10 weeks. Cultures were divided into two parts; one part was pelleted and dried for >2 hours in a vacuum oven at 100°C and the other part was used for the MTBE extraction. Dry weights of extracted lipids were compared with dry weight of the total culture.

***Electron microscopy*:** Cultures one week old were harvested from SGI plates for observation using electron microscopy. For scanning electron microscopy (SEM), samples were placed in 2% glutaraldehyde and 0.1 M sodium cacodylate buffer for 2 hours, rinsed in 0.1 M sodium cacodylate buffer, then placed in 1% osmium tetroxide and 0.1 M sodium cacodylate buffer for 2 hours. Specimens were rinsed in ultrapure water (NANOpure Infinity®; Barnstead/Thermo Fisher Scientific; Waltham, Maryland) and dehydrated in an ethanol series. After the samples were in 100% ethanol, they were put through two changes of hexamethyldisilazane (HMDS) for 5 min each. Drops of the suspension were placed on individual acetone-cleaned round glass cover slips that had been mounted on aluminum stubs with double-sided carbon adhesive tabs, and allowed to air dry. The specimen stubs were sputter-coated with gold-palladium and observed in a Hitachi S3500N scanning electron microscope at an accelerating voltage of 12 kV.

For transmission electron microscopy (TEM), samples were fixed as for SEM. Following the ultrapure water rinse, the samples were embedded in low melting point agarose. The samples were cut into 1-mm^3 pieces, dehydrated in

an ethanol series, and embedded in Embed 812 resin (Electron Microscopy Sciences, Hatfield, Pennsylvania). Ultrathin sections 80–100 nm thick were cut on a Leica Ultracut UCT microtome using a diamond knife and collected on formvar/carbon-coated copper mesh grids. Sections were post-stained with 3% uranyl acetate followed by Sato's triple-lead stain (Sato 1968), and examined with an FEI Phillips CM 12 transmission electron microscope operating at 60 kV. Images were recorded with a Maxim DL digital capture system.

Desiccation and freezing treatments: SGI Agar plates with healthy *H. coloradii* cultures were allowed to air-dry over a period of six months. After complete desiccation, cells were scraped from the plates onto fresh SGI plates to test for growth.

H. coloradii on BG-11 plates were placed in darkness in -20°C freezers for 2 weeks to test for freezing tolerance. After 2 weeks, plates were taken out to test for growth at either 4°C or 22°C in the light.

RESULTS

Biological composition of snow sample: Six different species of algae and bacteria were recovered from the snow sample. Two species of bacteria and four species of eukaryotic algae were identified.

In addition to *H. coloradii,* the samples were found to contain: *Pseudomonas antarctica*, 99% identity from BLAST with PCR-amplified 16s

rDNA (Genbank accession: BankIt1479737 seq JN661817); *Rhizobium sp.,* 98% identity from BLAST with PCR-amplified 16s rDNA (Genbank accession: BankIt1479737 seq. JN661816); *Chlamydomonas reinhardtii*, 100% identity confirmed via mating with a wild-type lab strain of *Chlamydomonas*; *Chlorella sp*. 99% identity from BLAST with PCR-amplified 18s rDNA (Genbank accession: BankIt1479737 seq2 JN661815).

Classification: *H. coloradii* was initially classified by the similarity of its morphology to other species of *Xanthophyceae*. Specifically, the cells had yellow-green chloroplasts, no starch, and swimming zoospores. Swimming cells have two flagella of unequal length (Fig. 1a). The long flagella is 7-10 μm long and the short flagella is 1.5-4 μm long. Morphology of *H. coloradii* is mostly congruent with reports of other species of *Heterococcus* from Antarctica and Europe (Lokhorst 1992) except for its unusually high lipid production and size. *H. coloradii* is the first species of *Heterococcus* to be discovered in North America.

Heterococcus normally grows as cell chains or in a coccoid form (Fig. 1e, 1f, 1g). We use the term “cell chains” instead of “filaments” so as not to be confused with fungal-type hyphae. *Heterococcus* cells are usually uninucleate (Lokhorst 1996) but our strain is multi-nucleate, containing 1-3 nuclei per cell (fig 2). A closely related genus, *Botrydiopsis*, is multinucleate and grows as large coccoid cells. The closeness of the morphology and molecular phylogeny of *H. coloradii* to *Botrydiopsis* might suggest that *H. coloradii* is actually a species of

Botrydiopsis. However, the presence of cell chains in *H. coloradii* alone is reason to classify it as a species of *Heterococcus.*

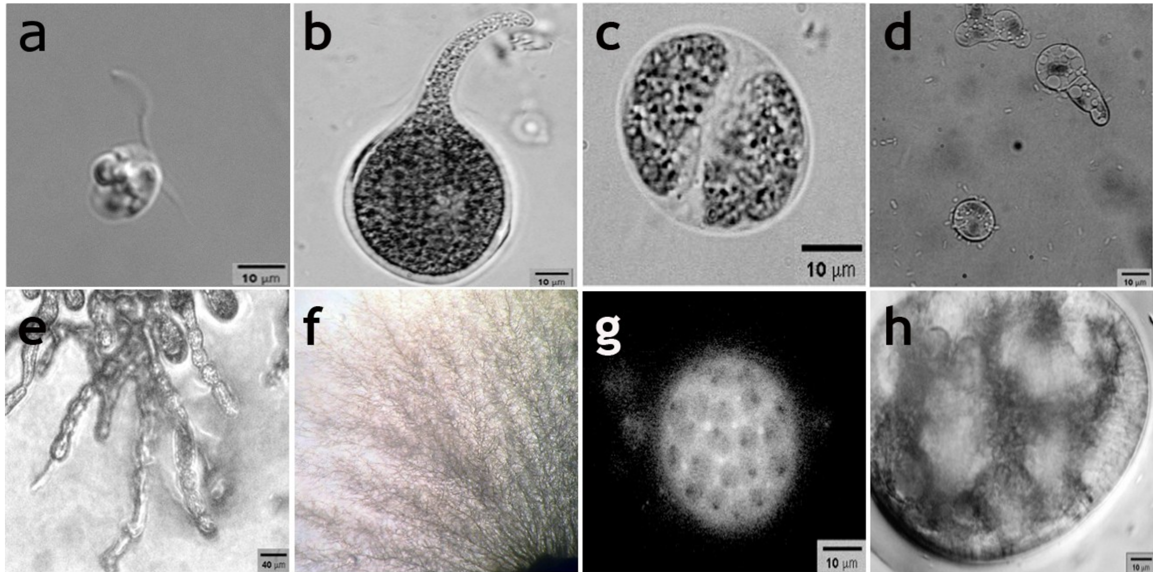

Figure 1: **Stereo microscopy (f) and DIC microscopy (a-d, g-h 1000x, e 250x) of *H. coloradii* cells.**

a: biflagellate swimming cell b: Coccoid *H. coloradii* budding off into cell chain growth. c: *H. coloradii* cell undergoing cytokinesis. d: *H. coloradii* in environmental sample shown with bacteria. e: *H. coloradii* cell chains penetrating into agar plates (viewed at 250x). f: extensive cell chain growth after >2 months on an agar plate (viewed at 2x). g: Fresh cell chain growth. h: Giant akinete formed after >3 months.

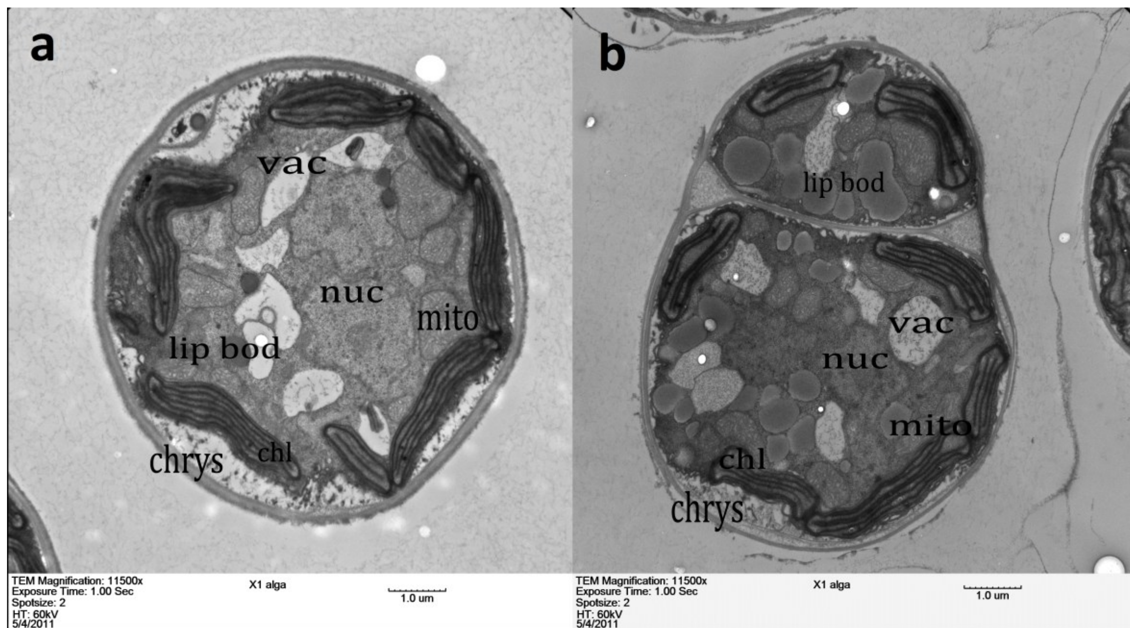

Fig. 2: Thin-section TEM of a) a non-motile coccoid cell and b) a dividing cell. Legend- nuc: nucleus (lower nucleus is labeled, organelle immediately above is also a nucleus), mito: mitochondria, lip bod: lipid body, chl: chloroplast, chrys: chrysolaminarin deposits.

DNA sequence analysis was used to confirm that the new species was of the genus *Heterococcus (*see *Molecular Methods).* BLAST searches with the PCR-amplified 18S rDNA (Genbank accession: BankIt1479737 seq1 JN661814) returned several species of *Heterococcus* with very low e values (Table 1). *H. chodatii* was recovered from Lake Geneva, Switzerland and is synonymous with *H. viridis. H. chodatii* displays isolated outgrowths of cell chains that are not seen in *H. coloradii. H. caespitosis* was recovered from Freiburg, Germany in wet, loamy soil. *H. caespitosis* is very similar to *H. coloradii* morphologically

except for *H. caespitosis's* distinct long cell chains that protrude from colonies. *H. fuornensis* was recovered from Fuorn, Switzerland from forest soil. *H. fuornensis* produces many immature coccoid cells from the ends of its cell chains that *H. coloradii* does not produce under any conditions. Although *H. coloradii* is similar these other species of *Heterococcus,* it is a unique species due to its large size, multinuclearity, and unusual oil accumulation.

Accession number	Description	E value	Max identity
AB534243.1	Uncultured eukaryote	1.00E-63	92%
AM490822.1	*H. chodatii*	1.00E-63	92%
AM490821.1	*H. fuornensis*	1.00E-63	92%
AM490820.1	*H. caespitosis*	1.00E-63	92%

Table 1: Identities of 4 top BLAST hits for PCR-amplified H. sp. 18S rDNA.

The consensus sequence from three of *H. coloradii's* closest relative differed from *H. coloradii* at 26/236 nucleotides (Fig.3). This significant nucleotide divergence of *H. coloradii* from its closest relatives is not surprising in view of the distance of the location of the recovered species (Colorado) to the published species (Antarctica). However, considering the sequence data

together with morphological data we concluded that the novel field isolate was nonetheless a species of *Heterococcus*.

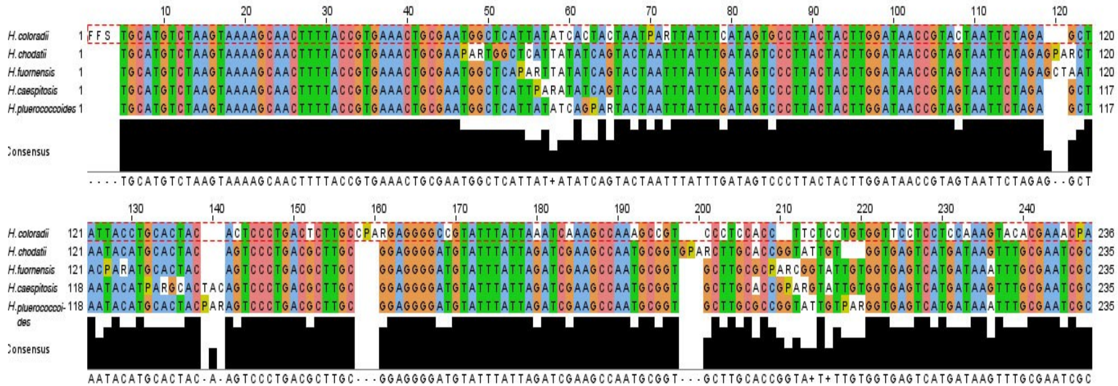

Fig. 3: Alignment of 18S rDNA amplified from genomic *H. coloradii* DNA with 4 closest relatives according to NCBI BLAST (*H. chodatii, H. fuornensis, H. caespitosis and H. pluerococcoides*) using ClustalW.

Lifecycle: *H. coloradii* cells can be found in a variety of forms, depending on age of the culture and growth conditions. Individual cells may be coccoid and immotile or may be swimming (figs. 1,5). Cells may be connected in cell chains or form giant akinetes (figs. 1,5). In liquid media *H. coloradii* divides via mitosis and maintains its coccoid form (Fig.1c). The age of the culture appears to govern what the cells morphology is. For the first week of growth, a newly streaked or inoculated culture will grow as coccoid cells. After one week, cell chains start to emerge. After two weeks, large spherical cells known as akinetes will appear and grow off the cell chains. Lipid accumulation commences after the first week of growth and can continue for up to six months.

When unperturbed by mechanical stirring, *H. coloradii* grows into branching cell chains (Fig.1e). These outgrowths are presumably to extend itself to gather nutrients. The chain growth occurs until the cultures reach higher cell densities. Photosynthetic cells deprived of nutrients lose chlorophyll although the more stable carotenoids will remain (Phadwal *et al.* 2003). When inoculated into fresh media the cells are dark green but as they use the available nutrients they shift to a bright yellow, perhaps as a consequence of nutrient depletion. As the cells change color, akinetes (Fig.1h) start to emerge. These are the cells that become completely engorged with lipids. Although all of the cell types of *H. coloradii* accumulate lipid bodies, the akinetes accumulate them to the highest degree and dedicate the largest intracellular volume to these bodies (Fig.4, top right cell in each panel).

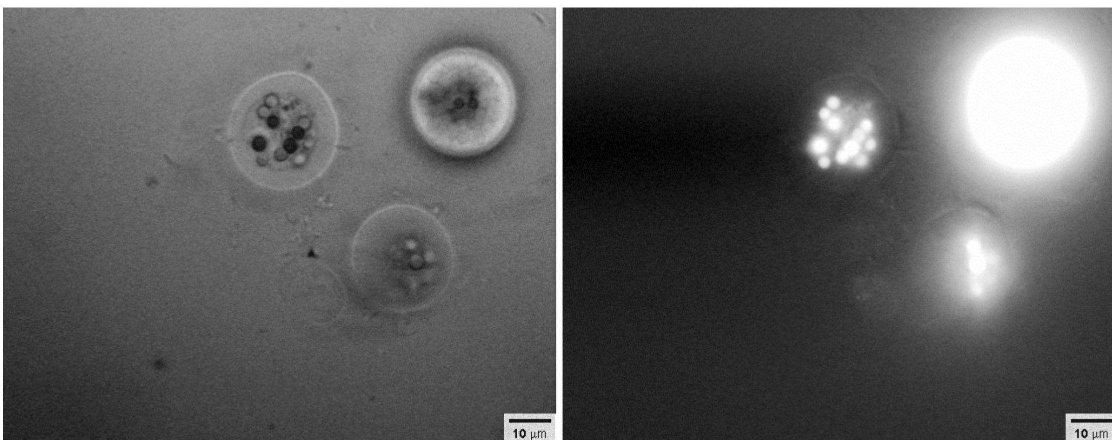

Fig. 4: 1000x DIC (left) and Nile Red fluorescence (right) images of *H. coloradii* showing oil accumulating as oil bodies (left and bottom cells) that eventually fill the intracellular space (upper right cell).

H. coloradii cells mature from a coccoid form to grow chains and then to form akinetes (Fig.5). Not all cells mature to form akinetes as some of the chain cells simply fill with lipids but never release zoospores. If the akinetes are inoculated into media with higher nitrogen levels they produce swimming zoospores that can number more than fifty per akinete. Zoospores may also contain large lipid bodies. Zoospores eventually stop swimming and mature into coccoid cells and the life cycle of *H. coloradii* starts anew (Fig.5).

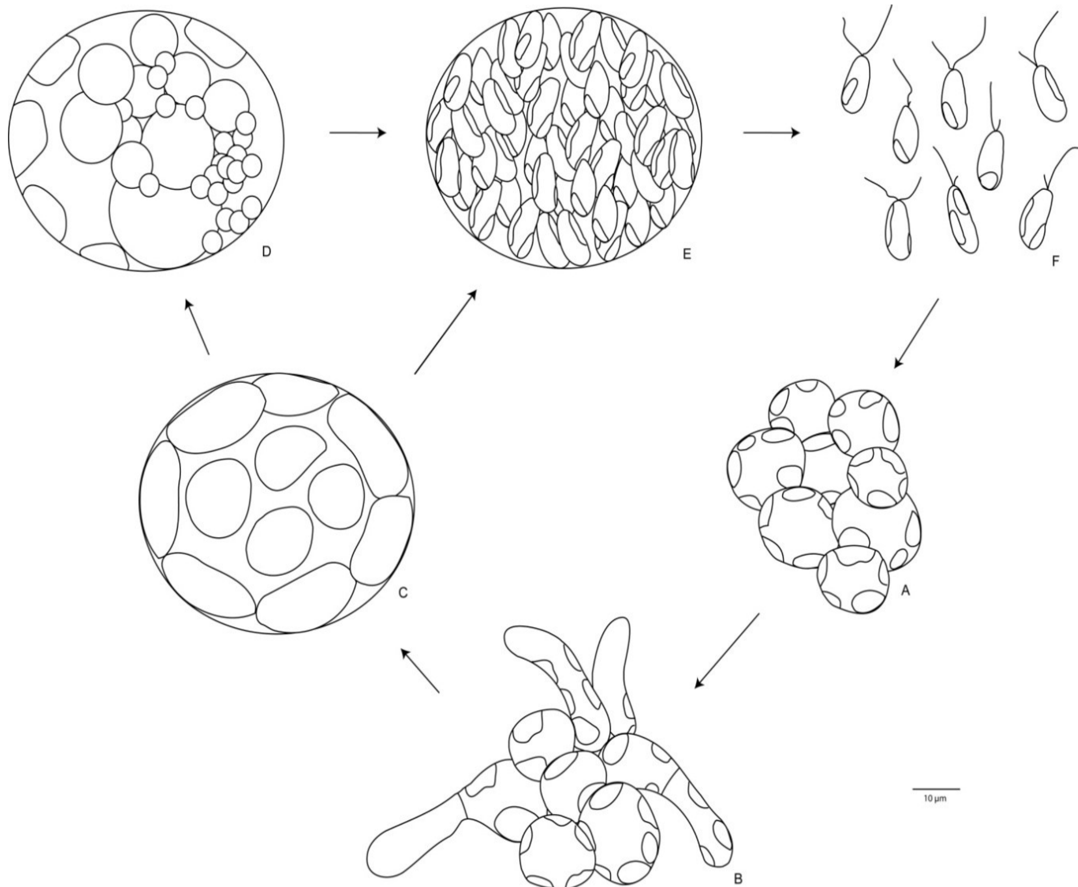

Fig. 5: Life cycle of *H. coloradii.* A: vegetative cell growth in coccoid form. Organelles shown are chloroplasts. B: Cell chain formation. C: akinete. D,

E: Cells produce lipid bodies(D) or zoospores (E) depending on nutrient availability. F: Swimming cells (zoospores).

Lipid accumulation: Lipid accumulation as visualized by Nile Red fluorescence was shown to fill up a majority of the intracellular volume. Lipids accumulate as spherical droplets that could range in size from 0.1 μm in diameter to 10 μm. Akinetes that had accumulated lipids could grow to larger than 100 microns in diameter (Fig. 1h). Fig. 4 shows mature cells with fluorescent lipid bodies stained with Nile Red. Lipids were determined gravimetrically using an advanced extraction method (see *materials and methods)*. On average a mature culture of *H. coloradii* is composed of 55% lipids by dry weight. Preliminary studies analyzing lipid composition using GC/MS indicated that certain high-value polyunsaturated fatty acids are present in significant amounts in *H. coloradii*.

Nutrients and Growth: Growth of *H. coloradii* was measured on several media designed for algal growth. Fig. 6 contains growth curves of *H. coloradii* for the four most effective media solutions used. Vegetative growth is characterized by cell division and chain growth, while non-vegetative growth is characterized by cell enlargement, akinete formation, and lipid accumulation. We found via flow cytometry that *H. coloradii* grew poorly in liquid media. Optimal growth was

observed on either filter paper or cellophane discs placed on top of the agar media. Although the main media commonly used to grow *Heterococcus* species is Bold's Basal Media (BBM, Lokhorst 1992), we found that SGI media was superior to other media tested in promoting rapid growth of *H. coloradii* (Fig.6). BG-11 media, however, was more effective at keeping cultures greener and viable for a longer time as observed during routine passing of cultures.

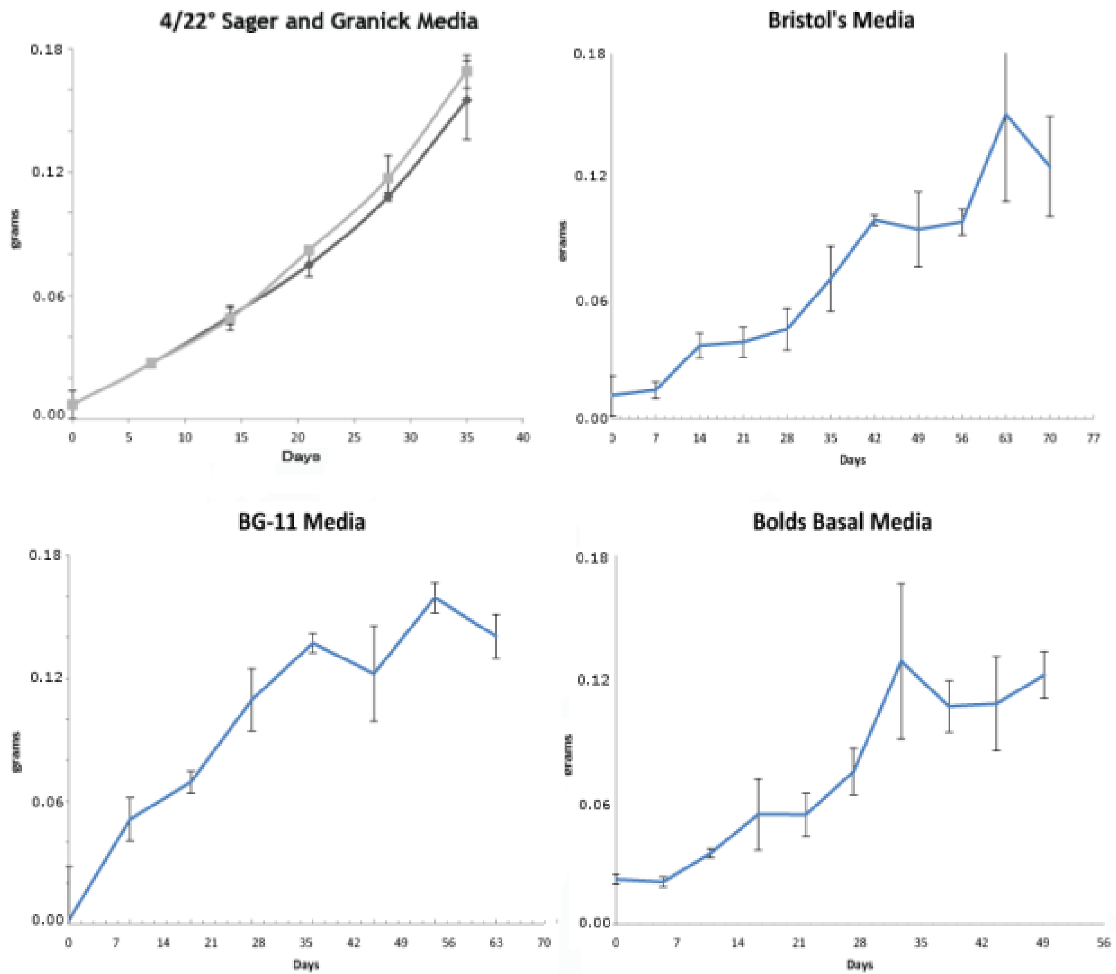

Fig. 6: Growth of *H. coloradii* was tested on different media. For SGI (M) chart: ■ = 22°C, ◆ = 4°C.   Weight is shown as total dry mass of *H. coloradii*.

Co-cultured bacteria: When *H. coloradii* was originally isolated as a single colony from the environmental sample, bacterial contamination was observed (Fig.1d, Fig.7). Two strains of bacteria were isolated (see *Biological composition of snow sample* section) and added back to axenic cultures of *H. coloradii.* The species of *Rhizobium* was found to have a significant physical interaction with *H. coloradii* as observed by SEM microscopy (Fig. 7) and was found to enhance growth of *H. coloradii* (Fig.8) as well as to increase viability under nitrogen deficient conditions (Fig.9).

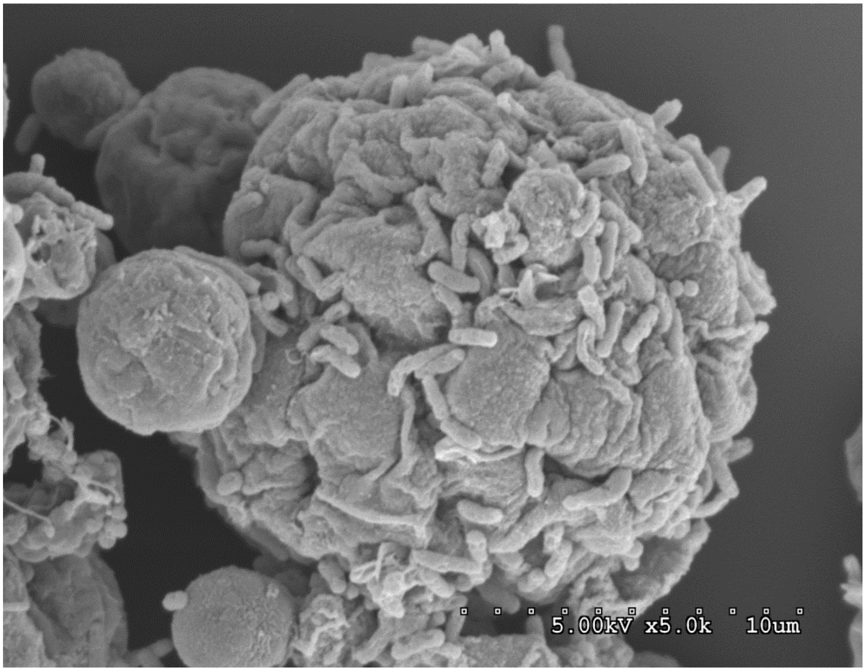

Fig. 7: SEM image of *Rhizobium sp.* attached to *H. coloradii* cell.

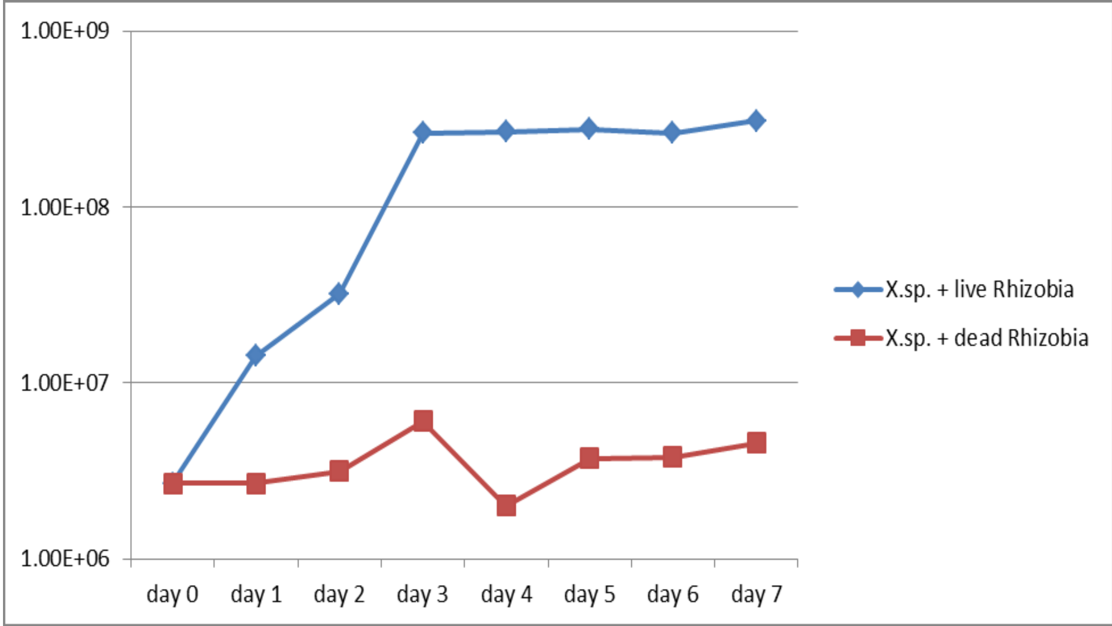

Figure 8: Growth of axenic *H. coloradii* compared with *H. coloradii* with *Rhizobium* added (appx. one toothpick per 2 ml well)

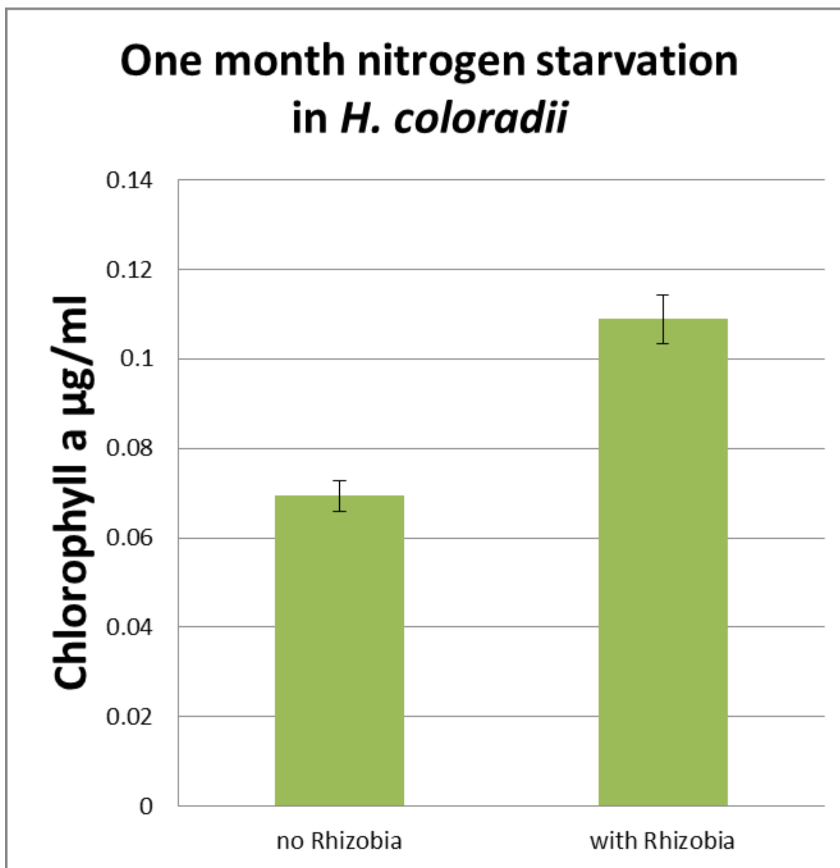

Fig. 9: Chlorophyll content of *H. coloradii* cells after one month of nitrogen starvation with and without co-cultured *Rhizobia.*

Desiccation and freezing survival: Some species of algae are known to survive long periods of desiccation (Gray *et al.* 2007). This capability, presumably an adaptive response to environmental conditions faced by the alga, could be useful for various commercial applications. We found that *H. coloradii* shows substantial tolerance to desiccation and cultures grow readily after desiccation treatments (see Materials and Methods*).* Desiccated cells are shown in Fig.10 to remain intact and maintain their lipid stores. *H. coloradii* was found

to survive periods of dark at -20°C. However, while *H. coloradii* could recover from freezing if placed at 4°C, cultures could not recover from freezing if they were thawed at 22°C.

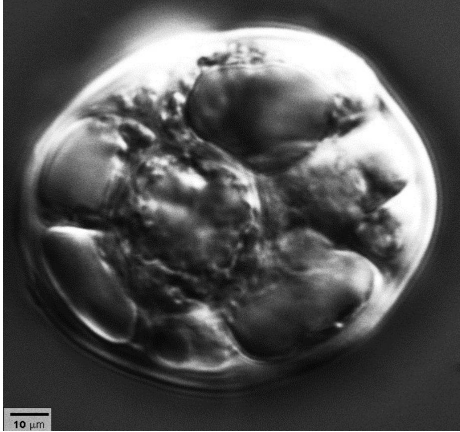

Fig. 10: Intact dessicated *H. coloradii* cell shown at 1000x DIC magnification

DISCUSSION

Reports on *Xanthophyceae* species are sparse in the scientific literature and many species are yet uncharacterized or entirely unknown (Ettl 1978). In fact, even the phylogenic distinctions of the genera within the Xanthophyta are dubious, and much debate surrounds the polyphyletic groupings of species (Maistro *et al.* 2009, Ehara *et al.* 1997). Despite the ambiguity surrounding the yellow-green algae phylogenetic classification system we have placed the newly discovered species into the genus of *Heterococcus* and give it the species name of *coloradii* because of the molecular data and its location of origin.

H. coloradii was found to exhibit a variety of morphologies depending on the age of the culture and environmental conditions. As *H. coloradii* grows it creates structures that withstand harsh environmental conditions such as freezing and/or drying. Once nutrients are restored, spores swim out and away from colonies for dispersion.

The new species of *Rhizobium* found with *H. coloradii* attaches to the outer cell wall and may promote growth. It is yet unknown exactly how the *Rhizobium* would promotes the growth of *H. coloradii* but options to speculate upon include: possible nitrogen fixation by the *Rhizobium,* possible vitamin supplementation provided by the *Rhizobium*, or the extra CO_2 provided by the *Rhizobium* is needed in an aquatic environment. Although acetylene reduction assays have been negative, it is possible that the proper environmental conditions for nitrogen fixation by an *H. coloradii*/*Rhizobium* consortium have not yet been found. Promotion of algal growth was found in early studies with flow cytometry but has been unable to be reproduced in future studies.

We are particularly interested in what *H. coloradii*'s lipid accumulation could mean in a commercial context. Algae have great potential to produce food, fuel, and medicine (Rasala *et al.* 2011). Among the most promising commercial applications for algae are the production of lipids for biodiesel production (Christianson *et al* 2011, Craggs *et al.* 2011, Hannan *et al.* 2010, Stephenson *et al.* 2011, Wiley *et al.* 2011) and high value lipids for human nutrition (Cockbain *et al.* 2011, Dangat *et al.* 2011, Doughman *et al.* 2007, Dewey *et al.* 2011,

Hegarty *et al.* 2011, Rhodes *et al.* 2009, Yeste *et al.* 2011). *H. coloradii* produces large amounts of intracellular lipids, making it a candidate for commercial lipid production. In fact, cells appear to fill completely with lipids before dying, leaving intact lipid containers as their remains.

Algae that can grow in extreme conditions and still accumulate lipids are of great interest to industry. *H. coloradii* grows at 4 ^{o} C and accumulates large intracellular stores of lipids. Thus, countries in higher latitudes may be able to produce omega-3 fatty acids in the wintertime without fishing by growing *H. coloradii.*

One problem frequently cited in algaculture is the limited size of unicellular algae cells with many species never growing over 10 microns in diameter. *H. coloradii* matures to create akinetes that can grow to over 100 microns in diameter, thus providing much more cellular volume that can be filled with lipids.

In most species of algae, high cellular density causes significant shading due to high amounts of fucoxanthin and other pigments. Shading causes less light to reach individual cells and shaded cells produce less lipids and become malnourished. *Xanthophyceae* lack fucoxanthin (Stace 1991), thus dense cultures of *H. coloradii* are remarkably translucent compared with other algae. Light harvest may be more efficient in *Xanthophycae* cultures for photosynthetic lipid production.

Many established algal lipid production systems rely on the alga making lipids from a supplied organic carbon compound (Wang *et al.* 2009). However

supplying exogenous carbon increases cost and it does nothing to sequester atmospheric or power-plant produced CO_2. An alga that produces lipids completely photosynthetically would be ideal. *H. coloradii* photosynthetically produces lipids solely from atmospheric CO_2 and does not use any other exogenous carbon compounds for growth or energy storage.

It is unknown exactly how many algal species are yet to be discovered but undiscovered species have great potential to possess characteristics useful for commercial applications. Thus more effort should be placed on the discovery and characterization of new strains of algae.

Chapter 3: Lipid composition of *Heterococcus coloradii* and response to variations in temperature and light.

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index: EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PA: palmitoleic acid

***H. coloradii* is a recently discovered species of yellow-green algae (*Xanthophyceae)* that grows at low temperatures and produces large amounts of intracellular lipids. Lipids accumulated by *H. coloradii* were analyzed using gas chromatography and mass spectrometry (GC/MS) and found to contain high levels of palmitoleic acid (PA, 16:1) and eicosapentaenoic acid (EPA, C20:5). PA and EPA are known to provide substantial health benefits, making *H. coloradii* an attractive organism for producing these lipids for human nutrition. We established the environmental conditions needed for production of lipids in *H. coloradii*. Various environmental stresses including extended periods of darkness or heat lower the proportion of high-value lipids in *H. coloradii*. *H. coloradii* produces the highest quantity of high-value lipids when grown undisturbed with high light in low temperatures**.

Keywords: algae, oil, lipids, algal oil, cold-tolerant, dessication-tolerant

INTRODUCTION

The world is in need of new sources of omega-3 fatty acids for human health (Whelan and Rust 2006). Omega-3 fatty acids, in particular very-long-chain fatty acids (VLCFAs) such as eicosapentaenoic acid (EPA) and

docosahexaenoic acid (DHA), are currently provided to the human population by supplementing diets with fish oil. The fish used for extraction of VLCFAs are almost always ocean fish as farmed fish have much lower levels of VLCFAs (Worm *et al.* 2009). However, large scale human consumption of ocean fish is not sustainable because of the high levels of contaminants such as mercury in ocean fish and the risk of depleting the world's fisheries (Whelan and Rust 2006, Worm *et al.* 2009).

The *Xanthopyceae* have been noted as a group of algae that accumulate high amounts of nutritionally important VLCFAs (Lang *et al.* 2011). Most species within the *Xanthophyceae* do not accumulate starch (Belcher and Miller 1960). Instead they store energy as lipids, and some species fill up a large portion of their total volume with lipids (Broady 1976). *H. coloradii* is a species within the *Xanthophyceae* that accumulates high amounts of nutritionally important fatty acids when grown in low temperatures. Accumulated lipids in plants and algae are stored as triglycerides in lipid bodies that usually range from 0.5-2.0 μm in diameter (Huang 1996, Tzen *et al.* 1993, Ting *et al.* 1996, Shimada TL *et al.* 2008). We describe the nature of lipid accumulation by *H. coloradii* and the variance of lipid types with varying environmental conditions as well as the composition of stored triglycerides compared with total lipids.

MATERIALS AND METHODS

Culture growth: All cultures of *H. coloradii* were grown on minimal salts media in 12% agar. Media used as indicated was either Minimal media (M) for growing *Chlamydomonas reinhardtii* with or without nitrogen (Harris 1998) or BG-11 media (cyanobacteria media, http://www-cyanosite.bio.purdue.edu/media/table/BG11.html). All cultures were grown at 4 ºC or 22 ºC and light grown cultures were grown at 1200 lux. Cells were grown on cellophane layered on top of agar plates for ease of harvest. All studies of lipid production in *H. coloradii* were done from cultures grown on solid media.

Lipid extraction/ gravimetric measurement: Lipids were extracted from *H. coloradii* using methyl *tert*-butyl ether (MTBE) according to a refined extraction protocol (Matyash *et al.* 2008). Extracted lipids were weighed and compared to dry weight from an equivalent volume of the same culture for each experiment to determine percentage lipids by dry weight.

Fluorescence microscopy**:** *H. coloradii* cells were stained with Nile Red in DMSO (Wang *et al.* 2009) for at least 2 hours prior to imaging. Cells were viewed at 1000x and 400x with a Diaplan differential interference contrast/fluorescence microscope filtered for 468 nm light and captured with a Jenoptic digital camera. Scale bars were created from measurements made with a 10 µm Carl Zeiss stage micrometer. Previous reports indicate that 10 minutes was sufficient to stain lipid bodies in a variety of algae but we found that optimal

staining of *H. coloradii* lipid bodies required at least 2 hours, perhaps because of the large size of *H. coloradii* lipid bodies.

Fatty acid composition analysis (GC/MS): Algal cultures were scraped from plates and pelleted into 15 ml disposable plastic tubes (Falcon) by centrifugation at 2,000 RPMs on an IEC DPR 6000 centrifuge using a 949 rotor. Lipids were then extracted from the pelleted cells (Folch *et al.* 1957). Dried extracts were resuspended in 1 ml of 5% HCL-methanol and placed in a 90° C water bath for 1 h to saponify and methylate the fatty acids. After allowing the samples to cool to room temperature, 1 ml of water was added and the fatty acid methyl esters (FAME's) were extracted 3 times with 1 ml of hexane prior to GC analysis. Fatty acid methyl esters were extracted with multiple hexane washes followed by GC analyses with a fused silica capillary column (Supelco Omegawax, model 122-7032), 30 m x 0.25 mm inner diameter (ID) x 0.25 µm film thickness, and Hewlett-Packard Agilent 5890 GC system with flame ionization detector (FID). The temperature program was as follows: 50 °C with a 2 min hold; ramp: 10°C/min to 250 °C with a 15 min hold. Constant pressure of 20 psi was applied throughout the run of 37 min per sample. Analyses were initiated by injection of 1 µL of sample at a split ratio of 20:1 and injector temperature of 250 °C. The FID temperature was set at 300 °C with air and hydrogen flow rates of 433 and 37 ml/min. Specific fatty acid methyl esters were identified based on retention time using a reference standard purchased from Nu-Chek Prep, Inc. Fatty acids are

referred to using lipid number nomenclature C:D, where C refers to the carbon chain length and D refers to the number of double bonds within the carbon chain (Rigaudy 1979).

Triglyceride composition analysis: 2 grams (dry weight) of lyophilized *H. coloradii* (equivalent to 96h 4°C light treatment) was sent to Medallion Labs (9000 Plymouth Avenue North, Minneapolis MN 55427. 1-800-245-5615, (763) 764-4453 Fax: (763) 764-4010) for triglyceride analysis. The sample was analyzed using AOCS Method Ca 9f-57 (Table 1).

Transmission electron microscopy: Cultures were harvested after one week of growth from SGI plates for observation by electron microscopy. Samples were placed in 2% glutaraldehyde and 0.1 M sodium cacodylate buffer for 2 hours, rinsed in 0.1 M sodium cacodylate buffer, then placed in 1% osmium tetroxide and 0.1 M sodium cacodylate buffer for 2 hours. Specimens were rinsed in ultrapure water (NANOpure Infinity®; Barnstead/Thermo Fisher Scientific; Waltham, Maryland) and dehydrated in an ethanol series. The samples were then embedded in low melting point agarose. The samples were cut into 1-mm^3 pieces, dehydrated in an ethanol series, and embedded in Embed 812 resin (Electron Microscopy Sciences, Hatfield, Pennsylvania). Ultrathin sections (80–100 nm) were cut on a Leica Ultracut UCT microtome using a diamond knife and collected on formvar/carbon-coated copper mesh grids. Sections were post-

stained with 3% uranyl acetate followed by Sato's triple-lead stain (Sato 1968), and examined with an FEI Phillips CM 12 transmission electron microscope operating at 60 kV. Images were recorded with a Maxim DL digital capture system.

RESULTS

Lipid accumulation: Lipids accumulated in *H. coloradii* as the cultures grew and matured on agar plates. Small lipid bodies were seen to emerge after roughly 96 hours of growth and these bodies grew steadily for up to 3 months in culture. Accumulation of lipids was found to occur strictly by photosynthesis, as the addition of fixed carbon in the medium in the form of acetate, glucose or malate did not enhance lipid production.

In mature cultures (> 1 month old) cells contained very large lipid bodies up to 10 μm in diameter (Fig.1b/f). At what appeared to be maximum lipid accumulation, lipid droplets filled the intracellular volume (Fig. 1). The accumulation of lipids in *H. coloradii* represents an extreme of algal lipid accumulation in that most cells fill completely with lipids without any special treatments such as nutrient deprivation (-N, -P, -Cu, -Mg, -Fe^{3+}), temperature changes, light schedule changes, or addition of carbon compounds to the growth media.

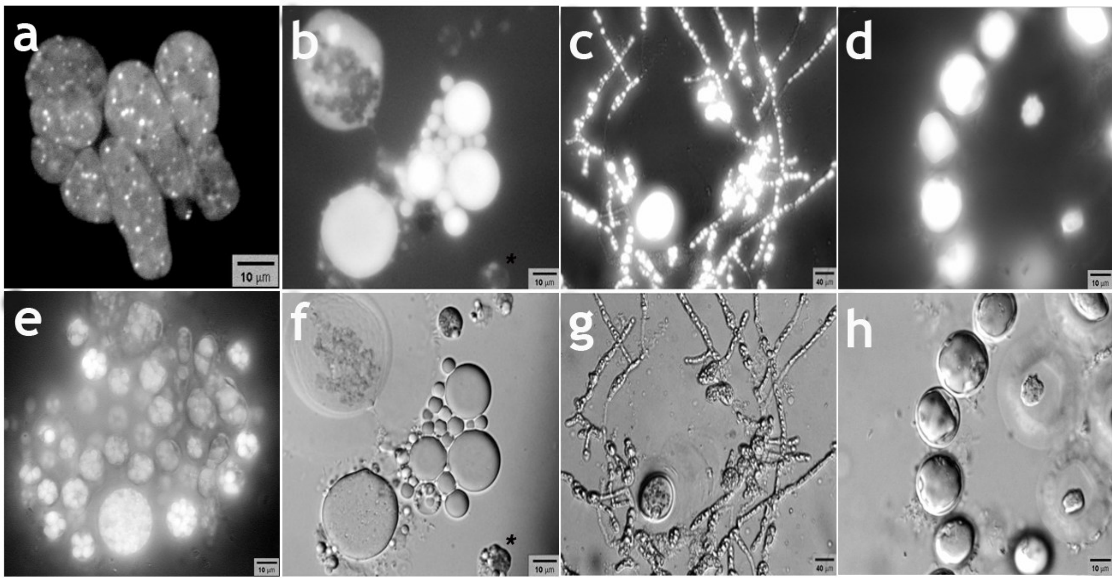

Figure 1: ***H. coloradii*** **cells viewed by DIC microscopy.**

a) Fluorescent Nile Red-stained lipid bodies beginning to form (100x). b) Lysed ***H. coloradii*** **cell filled with lipid viewed (100x) with fluorescence (b) or DIC (f)** ***Chlamydomonas reinhardtii*** **starch-less mutants** ***sta6*****, marked with stars, are shown for reference. c) 25x view of lipid-filled** ***H. coloradii*** **cells viewed with fluorescence (c) or DIC (g). d) Chain of** ***H. coloradii*** **cells filled with lipid viewed (100x) with fluorescence (d) or DIC (h). e) Group of lipid-filled** ***H. coloradii*** **cells.**

Cells ruptured easily under pressure on the cover slip and lipid bodies of different sizes and colors were released from *H. coloradii* cells (Fig. 1b/f). Lipids were not found to be released in the absence of applied mechanical force (e.g. as an inherent biological process). We conclude that the cells accumulate lipids

until they perish or become senescent and that the intracellular lipids continue to be stored within the confines of the cell indefinitely.

Composition analysis: GC-MS analysis showed that 16:1 and 20:5 fatty acids accumulated in cultures of *H. coloradii* as the dominant lipid species (Figures 2-5). To identify optimal conditions for producing these nutritionally-important fatty acids, experiments were conducted to observe how the levels of these fatty acids would change if *H. coloradii* cells were subjected to various environmental stresses. Among the stresses tested, temperature and light changes showed the most significant effect on fatty acid composition of *H. coloradii* cultures.

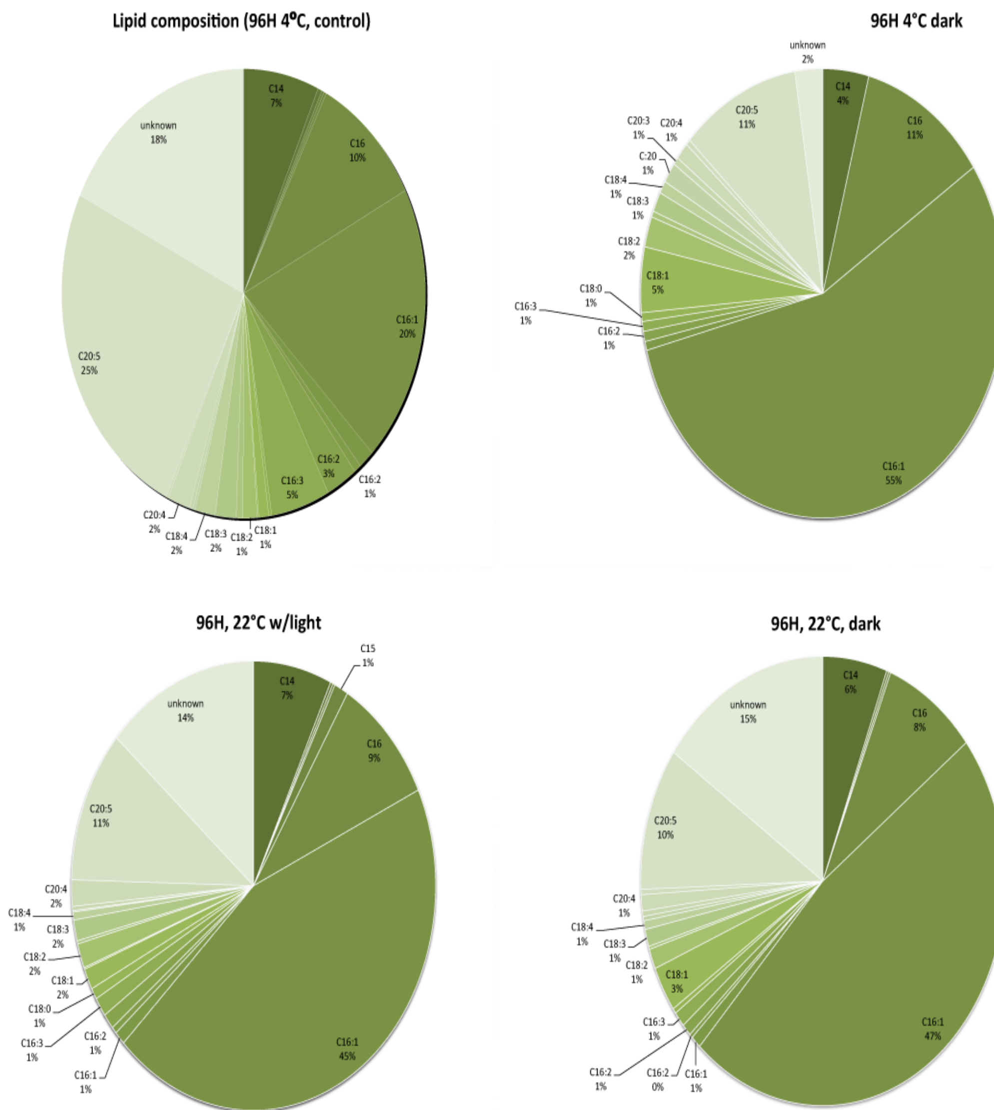

Fig. 2: Lipids in *H. coloradii* as determined by mass spectrometry. Lipid species named by length of carbon chain and number of double bonds: C16:3 has a 16-carbon length chain with 3 double bonds. *H. coloradii* cells were grown for one month at 4 °C at light levels of 1200 lux. Cells were given one of four treatments as indicated for 96 hours. After treatment,

cells were harvested and total lipids were analyzed by GC/MS. Any stress treatment served to lower the amount of EPA while total lipid amounts were not significantly affected.

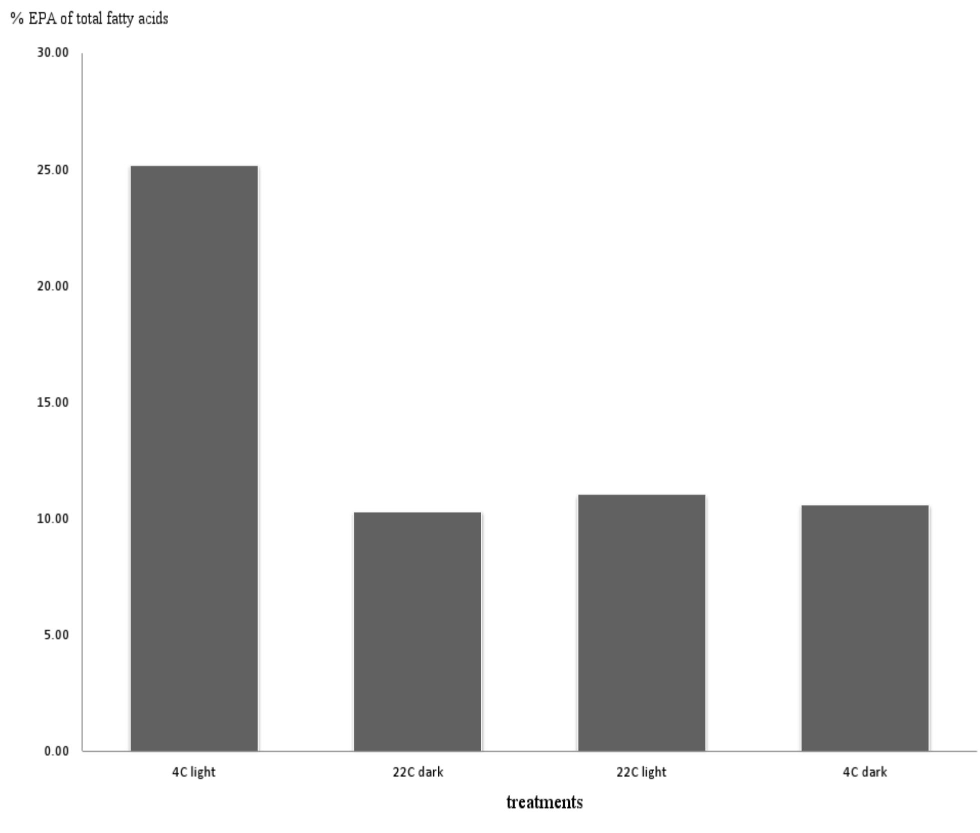

Fig. 3: **All stress treatments served to reduce the amount of percentage of EPA (C20:5) as a fraction of total lipids in *H. coloradii* cultures.**

The percentage of 16:1, palmitoleic acid (PA), was increased in all treatments. In cultures that received constant light at 4 ºC (control), PA levels stayed at 22%, but cultures that received dark treatment had 55% PA at 4 ºC and 47% PA at 22 ºC. Cultures that were moved to 22 ºC but still received light gained PA to 45% of their total lipids (Fig.2). EPA levels were decreased in all treatments. EPA levels fell from 25% to 10-11% when either moved to 22 ºC, moved to the dark, or received both treatments concurrently. Cultures that received dark treatment at 4 ºC had much lower levels of unidentifiable lipids (2% of total lipids) as opposed to all other cultures analyzed (15-18%). The reason for this drop in unidentifiable lipids is unknown.

Total lipid amounts were largely unchanged in all treatments as measured as a percentage of dry weight (55% lipids by dry weight). Also, no degradation of lipid bodies was observed with any treatments amounting to less than one week in duration. Normally when *H. coloradii* lipid bodies are degraded, pits can be clearly seen on the lipid body surfaces and no pits were seen in these experiments. The relative fluorescence level of Nile Red and Bodipy dyes can be used to quantitate lipid levels but previous studies have found that fluorescence levels do not necessarily correlate well with the fat levels detected by GC-MS (Brooks *et al.* 2009, *Zhang et al.* 2010).

No environmental treatment was shown to improve lipid accumulation, including high light, nitrogen starvation, bubbling CO_2-containing air into liquid cultures, and changing the pH of the media. Treatments of 96 hour heat stress

(4 °C to 22 °C), dark stress, or both heat and dark stresses applied concurrently changed fatty acid composition (Fig. 2). In short, EPA levels were significantly reduced with the introduction of stress while the levels of the 16-carbon fatty acids increased.

A detailed triglyceride composition analysis was obtained (Medallion Labs) in order to compare fatty acids in triglycerides to the total fatty acids in *H. coloradii* (Fig. 4*).* While *H. coloradii* was found to have 36.9% triglycerides by dry weight, we measured total lipids at 55% lipids by dry weight. Thus 33% of lipids in *H. coloradii* reside in species other than triglyceride. EPA was found to compose the highest percentage of fatty acids *not* esterified to a glycerol backbone (Fig. 4).

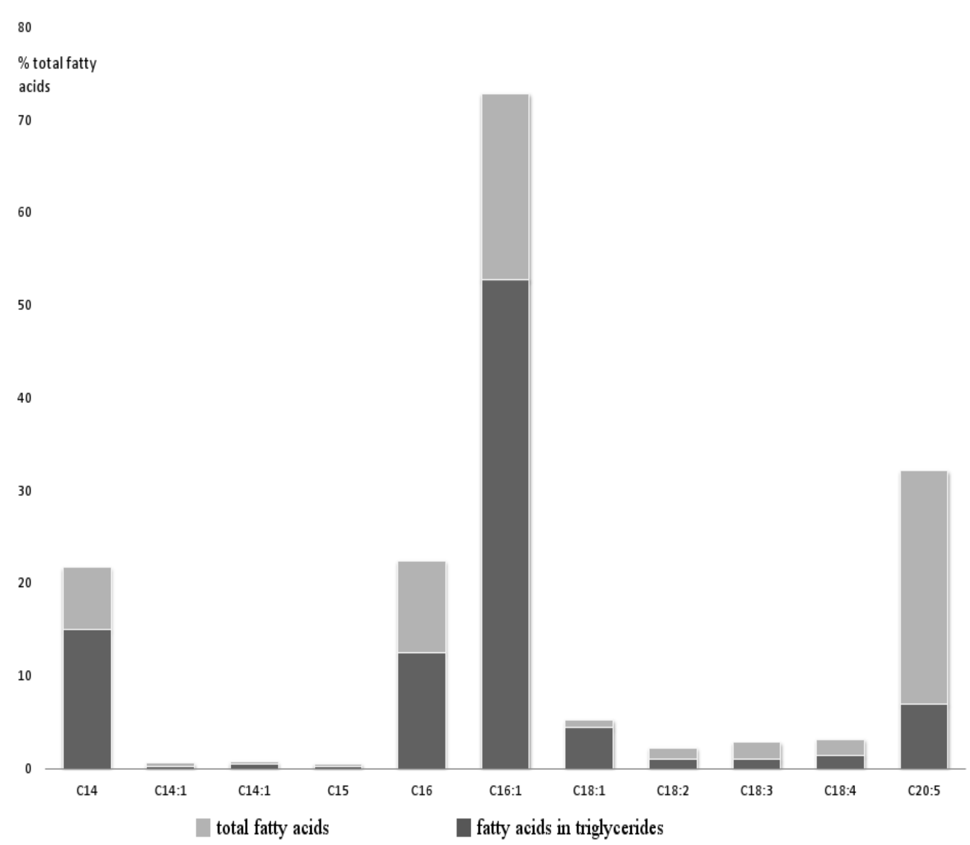

Fig. 4: **Difference in fatty acid composition of total triglycerides (dark bars) compared with total fatty acids (light bars).**

Ultrastructural analysis: Thin-section TEM revealed that lipid bodies in *H. coloradii* appear to be surrounded by an intracellular membrane (Fig.5). In other organisms lipid body membranes are composed of a phospholipid monolayer

(Shimada *et al.* 2008). Lipid bodies in *H. coloradii* did not appear to be associated with any particular organelle.

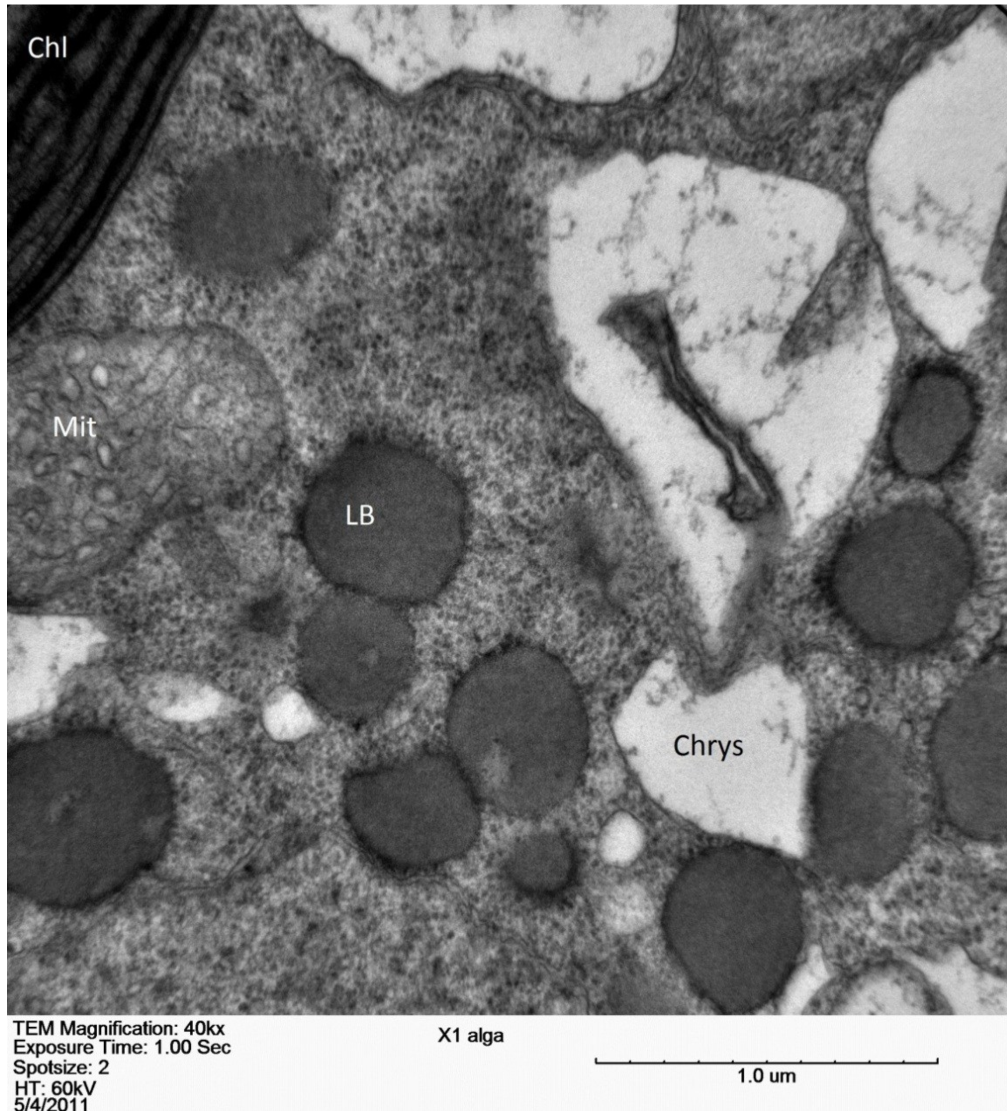

Fig. 5: **Thin-section TEM of *H. coloradii* cell highlighting small lipid bodies. LB: lipid body, Chrys: chrysolaminarin, Mit: mitochondria, Chl: chloroplast**

DISCUSSION

Algal strains for commercial production of biofuel will need to meet three criteria: 1) high growth rate, 2) high lipid content and 3) ease of harvest and extraction (Christiansen and Sims 2011, Craggs *et al.* 2011, De La Hoz *et al.* 2011). Often one or more of these criteria are not met for an otherwise desirable strain of algae. One solution is that different strains of algae will be cultured for different purposes. A strain of algae that produces high value omega-3 fatty acids may not be ideal for making jet fuel, and vice-versa. *H. coloradii* meets two of the three requirements listed above, but it grows fairly slowly. Its high lipid content should produce useful quantities of nutritionally valuable lipids, and its surface-growing lifestyle should be useful for harvesting lipid products without the need for energy-requiring de-watering steps.

The accumulation of lipids might serve several functions in *H. coloradii*. One obvious function is that of energy storage for long, dark, cold winters. Lipids are a particularly useful energy source where a slow, continuous energy supply with duration is needed. *H. coloradii* cells might spend the summer filling their intracellular spaces with lipids and slowly break them down over the wintertime. Cells might not be able to create photosynthetic energy due to the large amounts of snow coverage known to accumulate in the Rocky Mountains during the wintertime.

Also, the extensive lipid accumulation in *H. coloradii* may act to increase buoyancy. For non-swimming cells, buoyancy may mean the difference between

a substantial harvest of photons and CO_2 or starvation by darkness/anoxia at the bottom of a small pool of water. We have observed that *H. coloradii* does not grow well in liquid media. Thus it is possible that nature has selected for buoyant, lipid-rich cells of *H. coloradii* in aquatic environments.

EPA is necessary for membrane stability at lower temperatures (Kawamoto *et al.* 2009). For example, EPA-deficient Shewanella mutants have been shown significant growth retardation at 4 ^{o}C but not at 18 ^{o}C. In cold-grown *H. coloradii,* EPA was found to compose the highest percentage of fatty acids *not* esterified to a glycerol backbone. There are two possible reasons for this: 1) EPA is maintained in free fatty acid form at high levels *or* 2) EPA is maintained within the cell membrane as a polar lipid species at high levels. Since EPA is usually found in cell membranes in high levels (Kidd 2007), we presume this is also the case with *H. coloradii* and that the EPA in the cell membrane supports membrane stability.

Many health benefits of consumption of the very-long-chain omega-3 fatty acids (VLCFAs) EPA and docosahexaenoic acid (DHA) have been reported (Dewey *et al.* 2007, Hegarty and Parker 2011, Rhodes *et al.* 2009, Stebbins *et al.* 2010, Walser *et al.* 2006). These include the prevention of skin cancer (Rhodes *et al.* 2009), enhanced mood/decreased anxiety (Hegarty and Parker 2011), enhanced cardiovascular function (Stebbins *et al.* 2010, Walser *et al.* 2006), amelioration of cancer cachexia (Dewey *et al.* 2007), faster muscle recovery/anabolic effect (Smith *et al.* 2011), prevention of colorectal cancer

(Cockbain *et al.* 2011), prevention of atheroschlerotic plaques (Zampelas 2010), improved maternal lactation (Dangat *et al.* 2011), and improved sperm quality (Yeste *et al.* 2011). The World Health Organization recommends a dietary intake with a ratio of less than 10:1 of omega-6 fatty acids to omega-3.

Microalgae in general are known to have high levels of unsaturated fats that vary widely among species. Among the microalgae, cyanobacteria and green algae (Streptophyta and Chlorophyta) only accumulate low levels of lipids; these lipids are mostly saturated and monounsaturated lipid species. However, the Chromalveolate algae accumulate high levels of PUFAs(Watson 2003) that are comparable to *H. coloradii* lipid levels. *H. coloradii* accumulates the fatty acid EPA to as much as 25% of its total lipids. Microalgae usually have a ratio of 1:1 omega-6 fatty acids to omega-3 fatty acids (Lang *et al.* 2011).

Normally EPA and DHA can be supplied with fish oil, but using fish oil for DHA and EPA has a number of negative consequences such as overfishing the oceans and risk of contaminants (Worm *et al.* 2009, Bistrian 2010, Myers and Worm 2003). Purification of fish oil adds another high-cost process to the manufacture of a DHA/EPA-supplying product. Companies V-pure (Bedford, UK) and Deva (Chelsea AL, US) have already been established to supply DHA and EPA from algae as people are demanding a commercial alternative to fish oil for supplementation with DHA and EPA. Although the human body cannot readily synthesize adequate amounts of DHA or EPA, given enough EPA the body can easily synthesize enough DHA for its needs, and vice-versa (Rhodes *et al.*

2009). Also, there is evidence for specific health benefits for taking either DHA or EPA alone (Rhodes *et al.* 2009). Thus, it is important to discover species of algae that accumulate EPA and DHA alone.

As well as EPA, the fatty acid PA was also found to be abundant in *H. coloradii* (figs. 2,3, and Table 1). PA has been suggested to be a lipokine that is linked to whole body lipid metabolism (Cao *et al.* 2006). The authors found that PA was by far the most highly regulated fatty acid involved in adipose tissue metabolism. In adipocyte fractions of adipose tissue, PA, but not other lipids, down-regulated levels of pro-inflammatory cytokines. Thus there is evidence that PA may have therapeutic properties (Cao *et al.* 2006).

Component Name	% of lipids	% of dry mass	Saturated fatty acids	Monounsaturated fatty acids	Cis-cis Polyunsaturated fatty acids	Trans Unsaturated fatty acids
10:0 Capric	0.038	0.014	0.013			
12:0 Lauric	0.203	0.075	0.071			
13:0 Tridecanoic	0.035	0.013	0.012			
14:0 Myristic	15.007	5.537	5.246			
14:1 t-Tetradecanoic	0.239	0.088				0.083
14:1 Myristoleic	0.52	0.192		0.182		
15:0 Pentadecanoic	0.29	0.107	0.102			
16:0 Palmitic	12.524	4.621	4.403			
16:1 t-hexadecanoic	0.176	0.065				0.062
16:1 Palmitoleic	52.736	19.458		18.534		
17:0 Margaric	0.057	0.021	0.02			
17:1 Margaroleic	0.187	0.069		0.066		
18:0 Stearic	0.133	0.049	0.047			
18:1 Oleic	4.396	1.622		1.552		
18:2 Linoleic	0.9	0.322			0.318	
18:3 g-linoleic	0.528	0.195			0.186	
20:1 Gadoleic	0.295	0.109		0.105		
18:3 Linolenic	1.163	0.429			0.41	
18:2 conjugated Linoleic	0.038	0.014				
18:4 Octadeca-tetraenoic	1.388	0.512			0.49	
20:3 g-Eicosatrienoic	0.285	0.105			0.101	
20:4 Arachiodonic	0.287	0.106			0.102	
22:2 Docasadienoic	0.314	0.116			0.112	
20:5 Eicosapentaenoic	7.112	2.624			2.519	
24:1 Nervonic	0.463	0.171		0.165		
22:5 Docosapenta-enoic	0.043	0.016			0.015	
22:6 Docosahexa-enoic	0.06	0.022			0.021	
Totals:	100	36.9	9.91	20.76	4.28	0.19
% Fatty Acids			28.29	59.04	12.1	0.54

Table 1 : Summary of Medallion triglyceride analysis of *H. coloradii*.

In summary, *H. coloradii* has an excellent lipid profile for potential use as a source of lipids for human nutrition. The high amounts of EPA and PA together make this organism a very attractive potential food source. Mature cells might be harvested with little to no downstream processing and be sold as a nutritional supplement for human use.

Chapter 4: Lipid production and cold tolerance genes in *Heterococcus coloradii*

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A yellow-green alga, *Heterococcus coloradii,* was isolated from the snows of the Rocky Mountains in Colorado. *H. coloradii* displays unique properties such as abundant intracellular lipid accumulation and cold tolerance. Here we describe a number of genes discovered from a draft genome constructed from Illumina GAIIx short reads. *H. coloradii's* genome is 170 Mb, with 29,080 genes giving hits within NCBI using

TeraBlastP. We show that *H. coloradii* has a large number of genes involved in lipid metabolism and contains the required genes for the biosynthesis of eicosapentaenoic acid, a lipid required for nutrition in humans. A number of putative cold-tolerance-related genes are present in the genome as well.

INTRODUCTION

A species of *Heterococcus* was recently discovered that was found to accumulate large intracellular stores of lipids and grow at near-freezing temperatures. This psychrophilic algae accumulates the nutritionally important fatty acids eicosapentaenoic acid (EPA) and palmitoleic acid (PA) as its predominant lipid species, up to 25% and 55%, respectively. Because of interest into this unique organism's metabolism, a high throughput sequencing project was initiated.

Genomes from red, green and brown algae (Tirichine *et al.* 2011) and a transcriptome from a species of yellow-green algae, *Nannochloropsis* (Radakovits *et al.* 2012), have already been sequenced. However, experimentally verified genes in organisms closely related to *Heterococcus* are limited. *Ectocarpus siliculosis,* a brown alga, is the closest related organism with a completed genome (Cock *et al.* 2010).

To learn more about the complement of genes in *H. coloradii* and to characterize the genome of this species we sequenced its DNA. We sought to determine the size of *H. coloradii's* genome, make an estimate of the number of genes in *H. coloradii*, and determine possible biological processes in *H. coloradii* by homology of found genes with NCBI gene entries. We present here a subset of the discovered genes and how they characterize lipid production and cold tolerance in *H. coloradii.*

MATERIALS AND METHODS

H. coloradii cells were grown for 1 month on *Chlamydomonas reinhardtii* SG1 solid media (1.2% agar, Harris 1989) in 150X15mm agar petri dishes. Genomic DNA was harvested using the Gentra DNA extraction kit under conditions outlined by the manufacturer. More than 15 µg total DNA was extracted from *H. coloradii* using a Qiagen DNA extraction kit. Genomic DNA samples were quantified using a Pico Green Assay (Invitrogen). DNA samples were prepared for sequencing using the Illumina Genomic DNA Sample Preparation Guide.

Library preparation*:* Genomic DNA was fragmented using a nebulizer and compressed nitrogen. End repair was performed and the 3' ends were adenylated. Adapters were then ligated and the ligation products were gel

purified. Samples were then enriched for DNA fragments and gel purified again. The library was then validated and quantified using an Agilent High Sensitivity chip (Agilent Technologies), Pico Green Assay (Invitrogen) and KAPA qPCR (KAPA BioSystems).

Cluster generation: The Illumina cBOT was used for cluster generation. Flow cell and all clustering reagents were acquired from Illumina. The DNA template was immobilized to a random oligo lawn on the surface of a flow cell then amplified, linearized, and blocked. Sequencing primers were then hybridized to the template. Clustered flow cells were then loaded onto the Illumina GAIIx.

Data analysis: Read data was stored at the Minnesota Supercomputing Institute (MSI) as fastq files. Low quality scores were filtered and reads were trimmed at scores of 30 or above. Reads were assembled into contigs using the assembly program AbySS.

The best assembly was chosen for analysis of gene models. Preference was given to assemblies with the highest average contig size and number. For *H. coloradii,* an assembly using 54-bp fragments with 5-bp overlaps was found to give the best assembly (170 Mb genome size, 20x coverage). The 170 megabase assembled genome was organized into 78293 scaffolds to be used as a working *H. coloradii* genome draft (Table 2).

The assembled genome was then used for gene prediction using the computer software Genemark (GM, Borodovsky *et al.* 2011). Genemark ran trained on itself, *Chlamydomonas reinhardtii, Arabidopsis thaliana an*d *Medicago truncatula.* The final result was that Genemark found 37,288 predicted genes in the assembled genome of *H. coloradii.*

GM's prediction file (.hmm) was then used for a tera-BLASTp (http://www.ncbi.nlm.nih.gov/) in DeCypher (http://www.timelogic.com/catalog/755) to discover orthologous peptide sequences from NCBI.

RESULTS

61423467 total reads were organized into 102,129 contigs and 78,293 scaffolds (Tables 1,2) using the short read assembler ABySS (Simpson *et al.* 2009). K-mers, n-tuples of nucleic acids used to identify regions for assembly, of 52.5 bp were chosen to give the best assembly. The minimum contig length was 51, the average contig length was 1688.7, and the max contig length was 1688.7 bp. The minimum scaffold length was 52, the average scaffold length was 2213.6, and the maximum scaffold length was 100,893 bp. The genome was found to have a GC content of 48.69% (Table 1).

Name	OGT class	G/C %	Lineage
Xanthophyceae sp.	*P*	*48.69*	*Xanthophyceae*
Chlamydomonas reinhardtii	*M*	*64*	*Chlorophyceae*
Botryococcus Braunii	*M*	*54*	*Chlorophyceae*
Ectocarpus siliculosis	*M*	*30.7*	*Phaeophyceae*
Fucus vesiculosus	*M*	*28.9*	*Pheaophyceae*
Thalassiosira pseudonana	*M*	*47*	*Coscinodiscophyceae*
Ostreococcus taurii	*M*	*58*	*Chlorophyceae*
Chlorella variablis	*M*	*67.2*	*Trebouxiophyceae*
Cyanidioschyzon merolae	*T*	*55*	*Rhodophyceae*
Cyanidium caldarium	*T*	*53*	*Rhodophyceae*

Table 1: Comparision of GC content of *H. coloradii* with other species. P: Psychrophillic, M: Mesophillic, T: Thermophillic.

k-mer size (bp)	Contig #	Total bases in contigs	Contig min. length	Contig average length	Contig max. length	50% of contigs longer than:
52.5	102129	172461508	51	1688.7	78123	3996
Total bases in scaffolds	Scaffold min. length	Scaffold ave. length	Scaffold max. length	Total number of scaffolds		
173310572	52	2213.6	100893	78293		

Table 2: Description of final assembly of *H. coloradii* genome.

This assembly was used for gene predictions using Genemark. In total 37,288 genes were found. 88,079 peptides were predicted from Genemark's model. 64,303 hits were received from a Tera-BLASTP. 29,080 unique peptides were identified in *H. coloradii's* genome to be homologous with peptide sequences in NCBI with low E-values ($x<10^{-15}$).

We found that most hits came from *Ectocarpus siliculosus*, a brown macroalga (Table 3). Given that little is known about yellow-green algae, the finding of extensive similarities to brown algae may be valuable in analyzing gene function. Other organisms that shared a moderate number of proteins with *H. coloradii* included *Phytophora infestans*, or potato blight, with 1601 shared

proteins and *Thallasiosira pseudonana*, a diatom, with 864 shared proteins (Table 3). Only 298 protein hits were found comparing *H. coloradii* peptides to the the model alga *Chlamydomonas reinhardtii,* a chlorophyte.. Because the yellow-green algae are not well characterized molecularly we received few hits from this family.

Species	Description	Genome size (Mbp)	Top BLASTP hits from predicted peptides
Ectocarpus siliculosus	Brown alga	214	24734
Phytophthora infestans	Potato blight	250	1601
Thalassiosira pseudonana	Marine diatom	34	864
Oryza sativa	Rice	430	586
Volvox carteri	Multicellular alga	140	480
Physcomitrella patens	Moss	480	399
Aspergillus niger	Black mold	39	396
Chlamydomonas reinhardtii	Green alga "chlamy"	121	298
Dictyostelium discoidium	Slime mold	34	225
Neurospora crassa	Red bread mold	43	177
Vaucheria sp.	Yellow-green alga	N/A	95
Yarrowia lipolytica	Oil-rich yeast	20	71

Table 3: Comparison of organisms that received peptide matches in a BLASTP with translated nucleotide sequences from *H. coloradii.*

The overall functional gene composition of *H. coloradii* could be determined by using Gene Ontology (GO) terms. GO terms are descriptors used to describe functionally similar gene products (Torto-Alalibo *et al.* 2010, Hill *et al.* 2010). For example, kinases, transporters, etc. Using GO terms is a convenient way of describing biological processes in an organism (Hill *et al.* 2010). The goal of using GO terms was to categorize gene functions and perform broad functional annotation of *H. coloradii*'s modeled genes. Functional annotation incorporates experimental results from the research literature. Thus it is possible to functionally annotate predicted genes from *H. coloradii* using other organisms for which experimental results are available.

H. coloradii's putative proteome was used as source data for Blast2GO, a computer software program designed to annotate BLAST hits with GO terms and KEGG (Kyoto Encyclopedia of Genes and Genomes) metabolic pathway designations. Summaries of *H. coloradii's* genome in GO terms can be found in Fig.1. 505 proteins were found to have lipid metabolism-related functions.

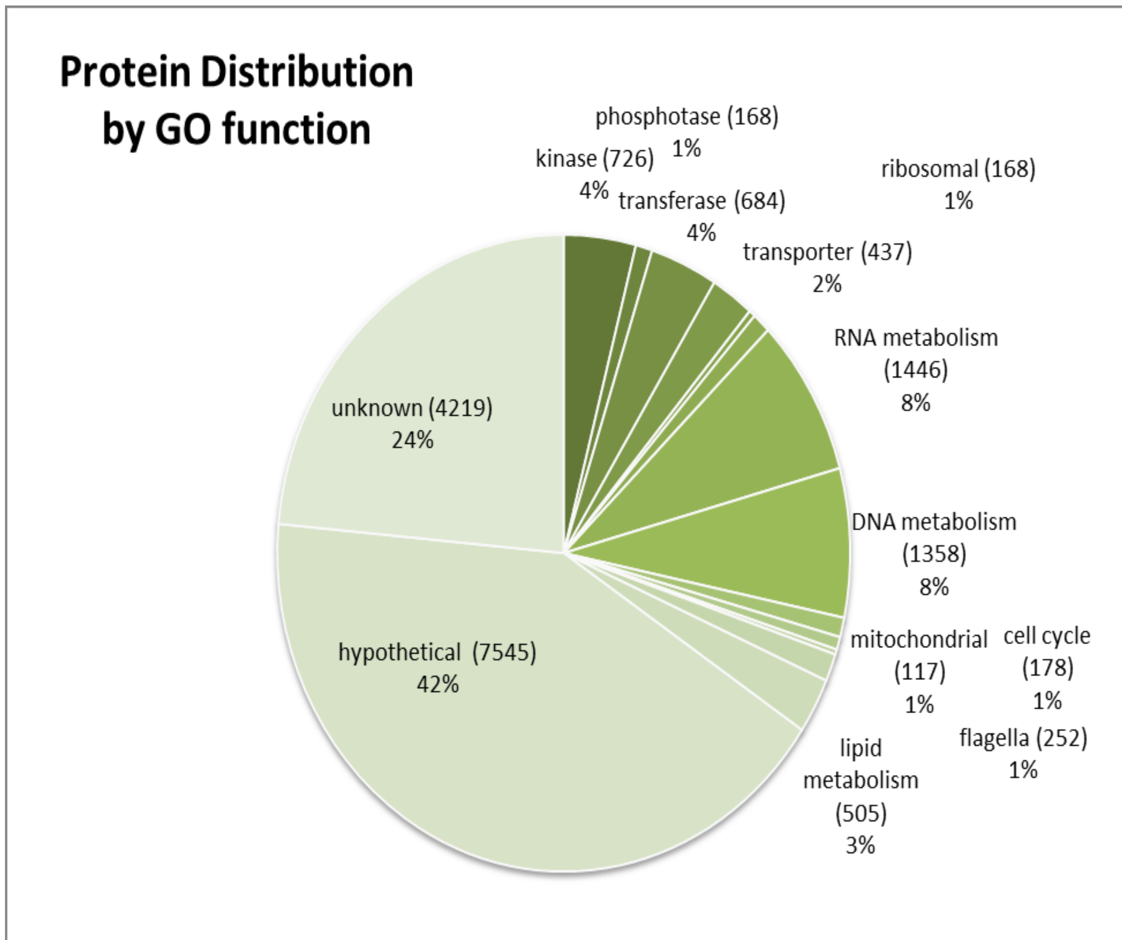

Figure 1: Composition of *H. coloradii's* genome regarding gene functional groupings (GO terms).

Proteins from *H. coloradii* were found to play a role in a number of different metabolic pathways as defined by KEGG. Pathways of interest include: chlorophyll metabolism, methane metabolism, fatty acid biosynthesis, and carbon fixation (Fig.2). *H. coloradii* appears to have extensive secondary metabolism that may be worthy of future investigation.

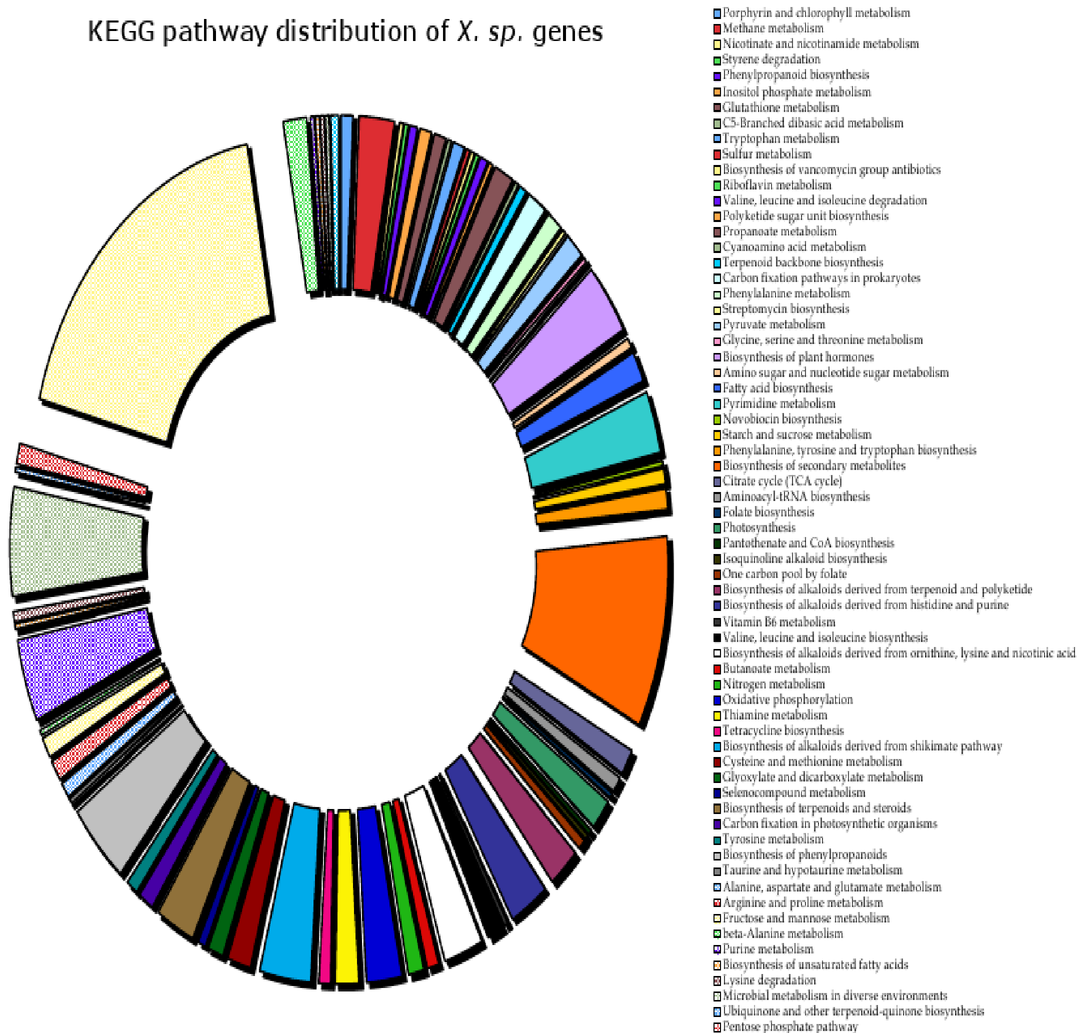

Fig. 2: **KEGG pathway distribution of *H. coloradii* genes (*www.genome.jp/kegg/*)**

Antifreeze proteins:

Since *H. coloradii* is native to a cold climate (0-30ºC) we hypothesized that it must have a number of antifreeze proteins (AFPs) to help it survive during the

winter months. A search through the draft genome revealed that there were 3 strong hits (E<-15) to AFPs in *H. coloradii* (figs. 3-5), one of which displayed the threonine array characteristic of certain ice-binding proteins (Davies 2002). AFPs were found from *Stigmatella aurantiaca*, *Notothenia angustata* (Maori Chief, cod icefish), which was a glycoprotein polyprotein, and from the filaria worm, *Bruglia malayi.* The AFP match from *Bruglia malayi* contained the threonine array.

```
X.sp/1-200          1 VQLRSASKYAVLAASTVTSTGFTTIKGLCSVSPGSEIVGFGPGTVNTLGMSATASIA 57
Stigmatella/1-203   1 VELGSAGNFAVMAGSTVTSTGQTSVTGDLGVSSGTAITGFPPGKLIGAKHAGDPTAA 57
Conservation
                      *8*4**869**9*+*******7*996*488**7*94*7**7**69677568+8797*
Quality
Consensus
                      V-L-SA---AV-A-STVTSTG-T---G---VS-G--I-GF-PG-------------A

X.sp/1-200         58 Q--RDAKLAYDDAAGRTVTELR-SGDLAGLTLFPGVYKSXXXXXXXXXXXXXXAQGD 111
Stigmatella/1-203  58 QGIADLTAAYNNAAGRSLCPVTVAGNLGGQTLTPGLYKSTSSLAISEGDLTLDAKGD 114
Conservation
                      *--4*767**88****998597-8*8*+*7**6**9***23323233342224*7**
Quality
Consensus
                      Q---D---AY--AAGR--------G-L-G-TL-PG-YKS-------------A-GD

X.sp/1-200        112 SDAVFIFQMASTLGTAAGRWVFLTGGAQAANVVWQVGSSCTLGIGSHLVGTVIAYSS 168
Stigmatella/1-203 115 GDAVFIFQMASTLTTTAGRQVVLTGGARSTNIFWQVGTSATFGTTSSFQGTVMADQA 171
Conservation
                      8************7*7***8*7*****887*97****9*9*8*67*486***9*588
Quality
Consensus
                      -DAVFIFQMASTL-T-AGR-V-LTGGA---N--WQVG-S-T-G--S---GTV-A---

X.sp/1-200        169 ITLTTGASVEGRLIALNGAVTLDSNVVTLPTP 200
Stigmatella/1-203 172 ITLNTGATLNGRALARIGAVALDDNTIVKPAP 203
Conservation
                      ***+***997**7+*56***7**7*7976*7*
Quality
Consensus
                      ITL-TGA---GR--A--GAV-LD-N----P-P
```

Fig. 3: ***H. coloradii*** **peptide sequence (pep48339) aligned with an antifreeze protein (ZP_01462925.1) from** ***Stigmatella aurantiaca*** **(49% identity).**

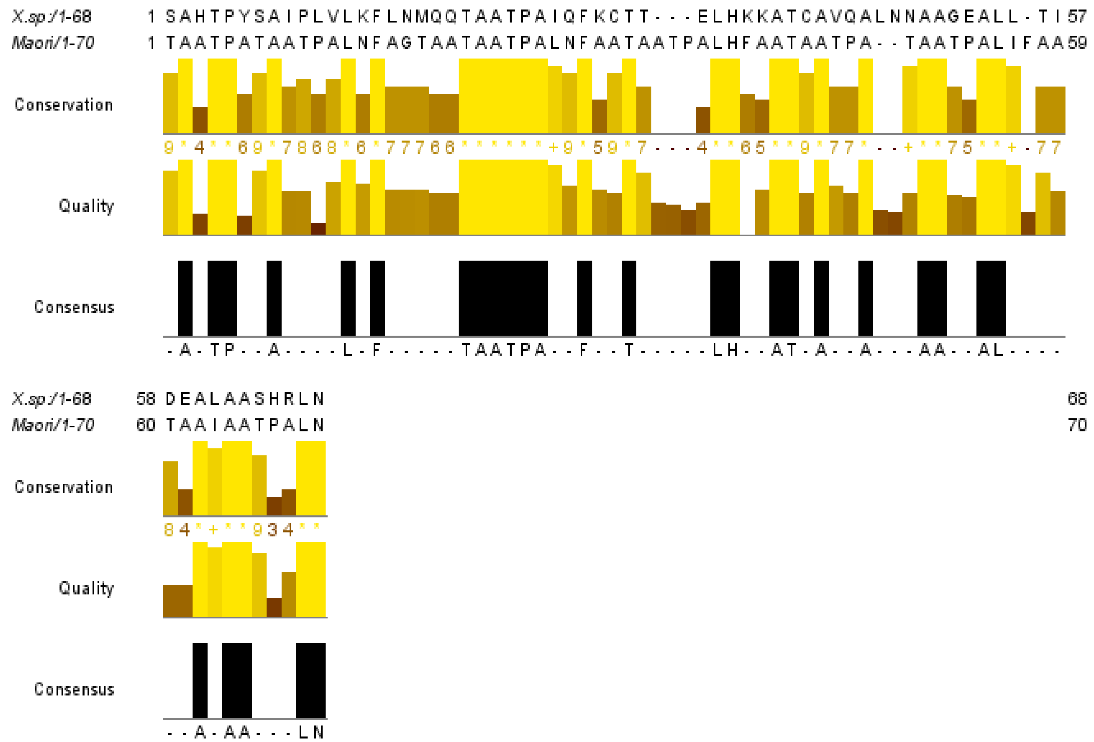

Fig. 4: **Alignment of *H. coloradii* peptide sequence (pep35355) with an antifreeze glycoprotein polyprotein (AAM61875.1) from *Notothenia angustata* (Maori Chief, cod icefish)n (40% identity).**

```
X.sp/1-66    1 TTAATATAAAAATAATTAATATAVAAATAAAVAAATAPAVAAAAAAAAVAVAATATLAIAV 61
Brugia/1-66  1 STAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 61

Consensus
               -TAA-A-AAAAA-AA--AA-A-A-AAA-AAA-AAA-A-A-AAAAAAAA-A-AA-A--A-A-

X.sp/1-66   62 TAAST                                                        66
Brugia/1-66 62 AAAIT                                                        66

Consensus
               -AA-T
```

Fig. 5: Alignment of *H. coloradii* peptide sequence (pep26099) with an AFP (XP_001892336.1) from the worm *Bruglia malayi* (65% identity).

Lipid metabolism:

BLASTP hits of *H. coloradii* peptides with proteins from other algae, fungi and some bacteria revealed more than 505 protein matches involved with lipid metabolism. BLASTP matches were found in organisms ranging from bacteria to gymnosperms.

Table 4 shows a summary of BLASTP matches found from *H. coloradii* peptide sequences that are involved with lipid synthesis and modification. *H. coloradii* has 6 peptides that match isoforms of diacylglycerol O-acyltransferase (CBJ30672.1, CBJ27686.1, CBN75192.1, XP_002906877.1, CBN77027.1, ABV91586.1, DGAT, EC:2.3.1.20). DGAT catalyzes the final reaction necessary to make triacylglycerol from diacylglycerol (Fig.6). Triacylglycerol is the main form of fatty acid storage in most organisms (Karantonis *et al.* 2009). In addition a number of proteins were found that are involved in nearly every step of lipid

synthesis from glycerol to mature triglycerides (Tables 4-6). Also included are enzymes that modify lipids by glycosylation or phosphorylation.

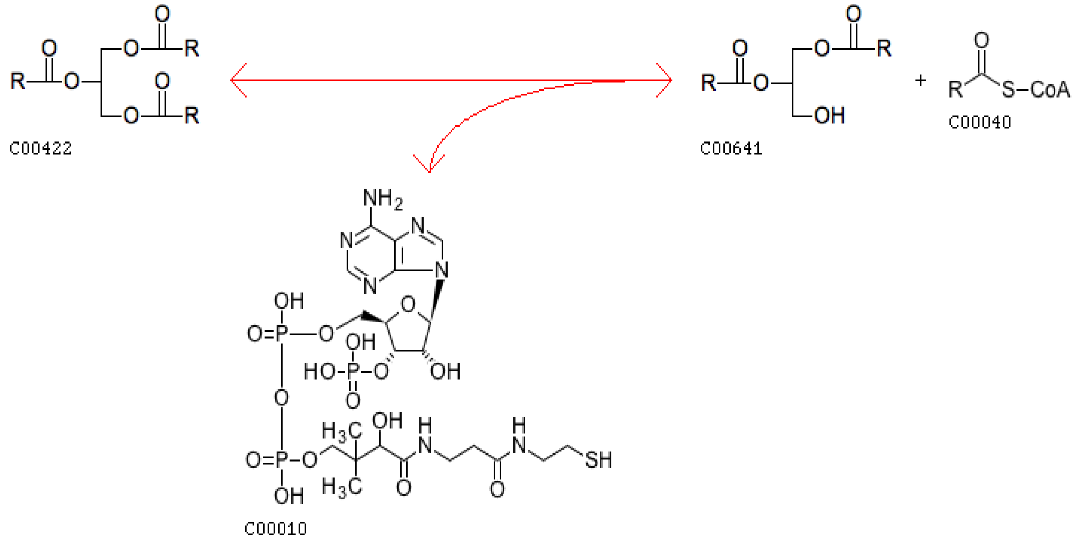

Fig. 6: Addition of third carbon chain to diacylglycerol by DGAT to form triacylglycerol. Image adopted from KEGG.

NCBI accession	Protein description
CBN77027.1	mono-or diacylglycerol acyltransferase type 2 [*Ectocarpus siliculosus*]
XP_642726.1	diacylglycerol kinase [*Dictyostelium discoideum*]
CBN75121.1	1-acyl-sn-glycerol-3-phosphate acyltransferase [*Ectocarpus siliculosus*]
CBJ28756.1	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatase [*Ectocarpus siliculosus*]

CBJ28372.1	Monogalactosyldiacylglycerol synthase, family GT28 [*Ectocarpus siliculosus*]
CBJ27648.1	Digalactosyldiacylglycerol synthase, family GT4 [*Ectocarpus siliculosus*]
CBJ28381.1	Monogalactosyldiacylglycerol synthase, family GT28 [*Ectocarpus siliculosus*]
CBN74380.1	diacylglycerol kinase (Partial) [*Ectocarpus siliculosus*]
AAG51624.1	putative phorbol ester / diacylglycerol binding protein [*Arabidopsis thaliana*]
CBN78066.1	1-acyl-sn-glycerol-3-phosphate acyltransferase [*Ectocarpus siliculosus*]
YP_003443466.1	1-acyl-sn-glycerol-3-phosphate acyltransferase [*Allochromatium vinosum*]
NP_001167366.1	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatase [*Salmo salar*]
CBJ29775.1	1-acyl-sn-glycerol-3-phosphate acyltransferase [*Ectocarpus siliculosus*]
CBJ30672.1	sterol or diacylglycerol O-acyltransferase [*Ectocarpus siliculosus*]
CBJ27686.1	mono-or diacylglycerol acyltransferase type 2 [*Ectocarpus siliculosus*]

CBJ27648.1	Digalactosyldiacylglycerol synthase, family GT4 [*Ectocarpus siliculosus*]
CBJ28381.1	Monogalactosyldiacylglycerol synthase, family GT28 [*Ectocarpus siliculosus*]
XP_002907046.1	1-acyl-sn-glycerol-3-phosphate acyltransferase [*Phytophthora infestans*]
CBN75192.1	mono-or diacylglycerol acyltransferase type 2 [*Ectocarpus siliculosus*]
ABV91586.1	diacylglycerol acyltransferase [*Zea mays*]
XP_002906877.1	diacylglycerol O-acyltransferase, putative [*Phytophthora infestans*]
CBJ25625.1	Glycerol-3-phosphate O-acyltransferase [*Ectocarpus siliculosus*]
CBN74441.1	CDP-diacylglycerol---inositol 3-phosphatidyltransferase [*Ectocarpus siliculosus*]
CBN77837.1	Diacylglycerol O-acyltransferase, type 1 [*Ectocarpus siliculosus*]
CBJ32618.1	diacylglycerol kinase [*Ectocarpus siliculosus*]
CBN74381.1	diacylglycerol kinase [*Ectocarpus siliculosus*]
CBN75531.1	mono-or diacylglycerol acyltransferase type 2 [*Ectocarpus siliculosus*]
CBJ27686.1	mono-or diacylglycerol acyltransferase type 2

	[*Ectocarpus siliculosus*]

Table 4: Enzymes involved in lipid synthesis and modification found with BLASTP from *H. coloradii* peptides.

Enzymes found that are involved in lipolysis are shown in Table 5. Among the enzymes found, triacylglycerol lipases are notable because they are responsible for the first committed step in triacylglycerol breakdown (Fig.7). Figs. 8-10 demonstrate the similarity between a *H. coloradii* peptide sequences and a lipases from various other species, including a triacylglycerol lipase (ZP_05344363.3) from the ant *Bryantella formatexigens.* Other notable enzymes found involved in lipolysis include phospholipid lipases; phospholipid lipases remove fatty acids from the membrane into storage.

NCBI accession	Protein description
CAL50026.1	Predicted lipase (ISS) [Ostreococcus tauri]
CBJ26506.1	lipase class 3 [*Ectocarpus siliculosus*]
CBJ26711.1	Putative lysophospholipase, monoglyceride lipase [*Ectocarpus siliculosus*]
CAL50026.1	Predicted lipase (ISS) [Ostreococcus tauri]
CBJ26506.1	lipase class 3 [*Ectocarpus siliculosus*]
CBJ27097.1	Lipase domain protein [*Ectocarpus siliculosus*]
CBJ29381.1	phospholipase A2, group VII [*Ectocarpus siliculosus*]

CBJ32519.1	lipase [*Ectocarpus siliculosus*]
CBN73949.1	Putative phospholipase [*Ectocarpus siliculosus*]
CBN75770.1	lipase, putative [*Ectocarpus siliculosus*]
CBN76512.1	similar to phospholipase C, Δ 4 [*Ectocarpus siliculosus*]
CBN76735.1	lipase [*Ectocarpus siliculosus*]
CBN77084.1	similar to Calcium-independent phospholipase A2-gamma [*Ectocarpus siliculosus*]
CBN77733.1	Lipase domain protein, partial [*Ectocarpus siliculosus*]
CBN79491.1	carboxyl-ester lipase [*Ectocarpus siliculosus*]
CBN79984.1	Putative phospholipase [*Ectocarpus siliculosus*]
NP_000291.1	phospholipase A2, membrane associated precursor [*Homo sapiens*]
NP_001135601.1	monoacylglycerol lipase ABHD12 [*Xenopus (Silurana) tropicalis*]
NP_009623.1	Major cell wall mannoprotein with possible lipase [*Saccharomyces cerivisiae*]
NP_631918.3	sn1-specific diacylglycerol lipase beta isoform 1 [*Homo sapiens*]
XP_001178303.1	phospholipase [*Strongylocentrotus purpuratus*]
XP_001377685.1	phospholipase A2 [*Monodelphius domestica*]
XP_001831193.1	lipase [*Coprinopsis cinerea okayama7#130*]
XP_001853511.1	hepatic triacylglycerol lipase [*Culex quinquefasciatus*]

XP_002129990.1	phospholipase C, Δ 4 [*Ciona intestinalis*]
XP_002193959.1	patatin-like phospholipase domain containing 1 [*Taeniopygia guttata*]
XP_002340899.1	lipase/esterase [*Talaromyces stipitatus*]
XP_002748329.1	phospholipase D2 [*Callithrix jacchus*]
XP_002896651.1	patatin-like phospholipase [*Phytophthora infestans*]
XP_002999064.1	patatin-like phospholipase [*Phytophthora infestans*]
XP_003009439.1	cytosolic phospholipase A2 zeta [*Verticillium albo-atrum*]
XP_811442.1	lipase [*Trypanosoma cruzi strain CL Brener*]
YP_422353.1	glyoxysomal fatty acid beta-oxidation multifunctional protein MFP-a [*Magnetospirillum magneticum AMB-1*]
YP_001196580.1	esterase/lipase-like protein [*Flavobacterium johnsoniae*]
YP_001302072.1	putative patatin-like phospholipase [*Parabacteroides distasonis*]
YP_001703547.1	lipase LipH [*Mycobacterium abscessus ATCC 19977*]
YP_002127564.1	phospholipase/Carboxylesterase [*Alteromonas macleodii*]
YP_003249593.1	putative esterase/lipase/thioesterase [*Fibrobacter succinogenes*]
YP_003527017.1	lipase class 3 [*Nitrosococcus halophilus Nc4*]
YP_003713434.1	FliA regulated lipase [*Xenorhabdus nematophila*]
YP_131916.1	putative phospholipase [*Photobacterium profundum*]

YP_159518.1	putative phospholipase [*Aromatoleum aromaticum*]
YP_357523.2	phospholipase D protein, putative [*Pelobacter carbinolicus*]
YP_585935.1	triacylglycerol lipase [*Cupriavidus metallidurans*]
ZP_00683171.1	Triacylglycerol lipase [*Xylella fastidiosa Ann-1*]
ZP_02376724.1	probable phospholipase C (plcA) [*Burkholderia ubonensis*]
ZP_02731213.1	Esterase/lipase [*Gemmata obscuriglobus UQM 2246*]
ZP_05344363.3	triacylglycerol lipase [*Bryantella formatexigens*]
ZP_06488249.1	putative phospholipase accessory protein [*Xanthomonas campestris*]
CAL50026.1	Predicted lipase (ISS) [*Ostreococcus tauri*]

Table 5: Enzymes involved in lipolysis found with BLASTP from *H. coloradii* peptides.

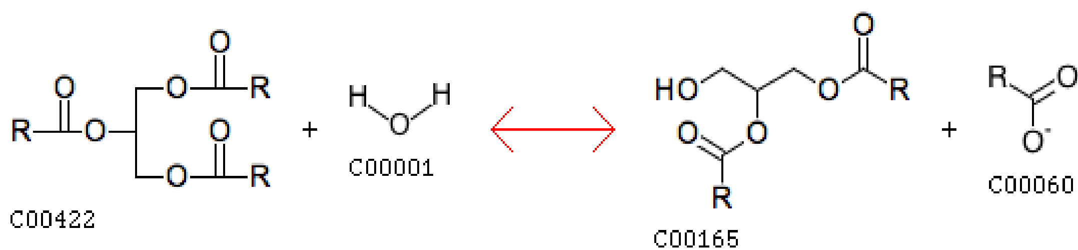

Figure 7: Triacylglycerol lipase (EC: 3.1.1.3) catalyzes the first committed step of triacylglycerol breakdown (Fig.adapted from KEGG)

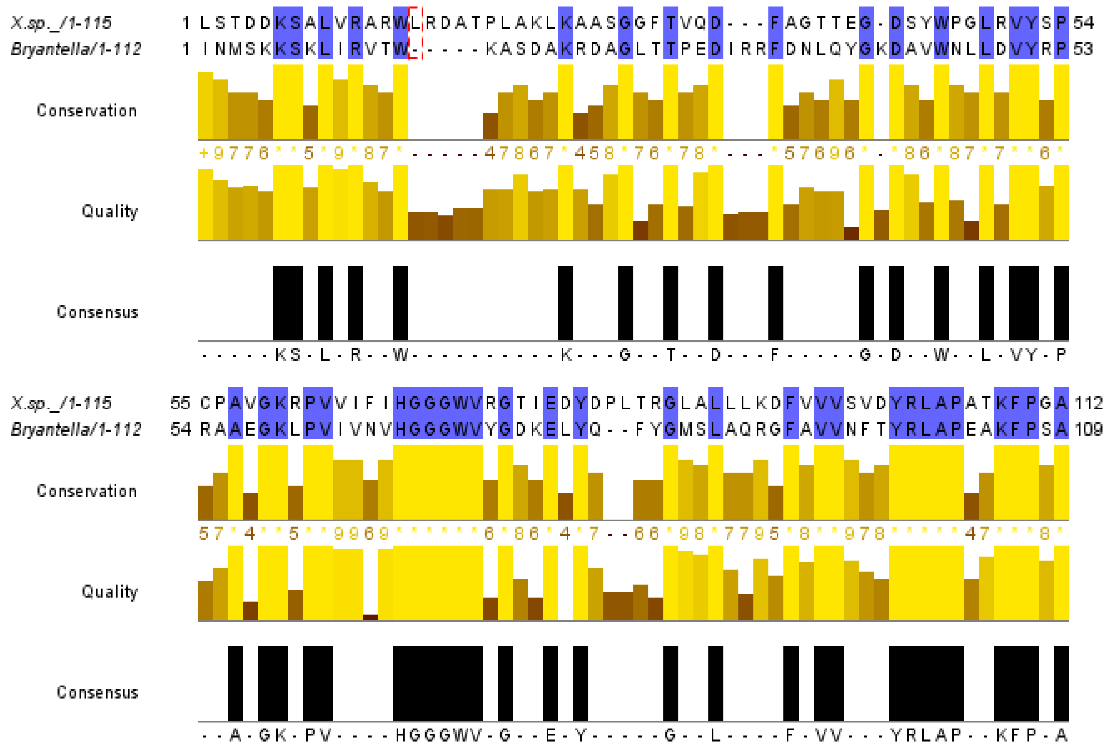

Fig. 8: Alignment of *H. coloradii* peptide sequence (pep24031) with a triacylglycerol lipase (ZP_05344363.3) from the ant *Bryantella formatexigens (*38% identity)*.*

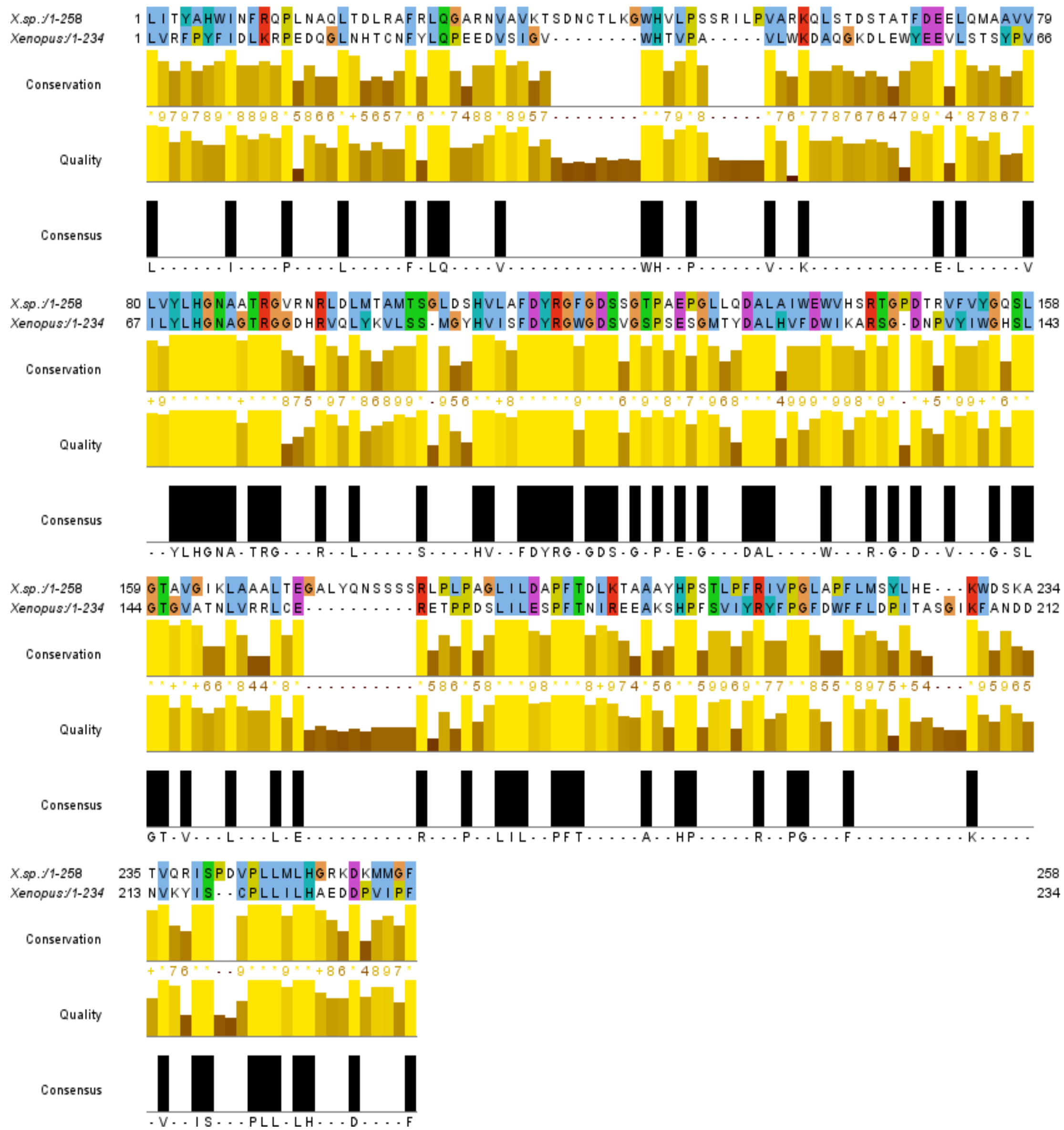

Fig. 9: Alignment of *H. coloradii* peptide sequence (pep18689) with a monoacylgylcerol lipase (NP_001135601.1) from the frog *Xenopus silurana* (32% identity).

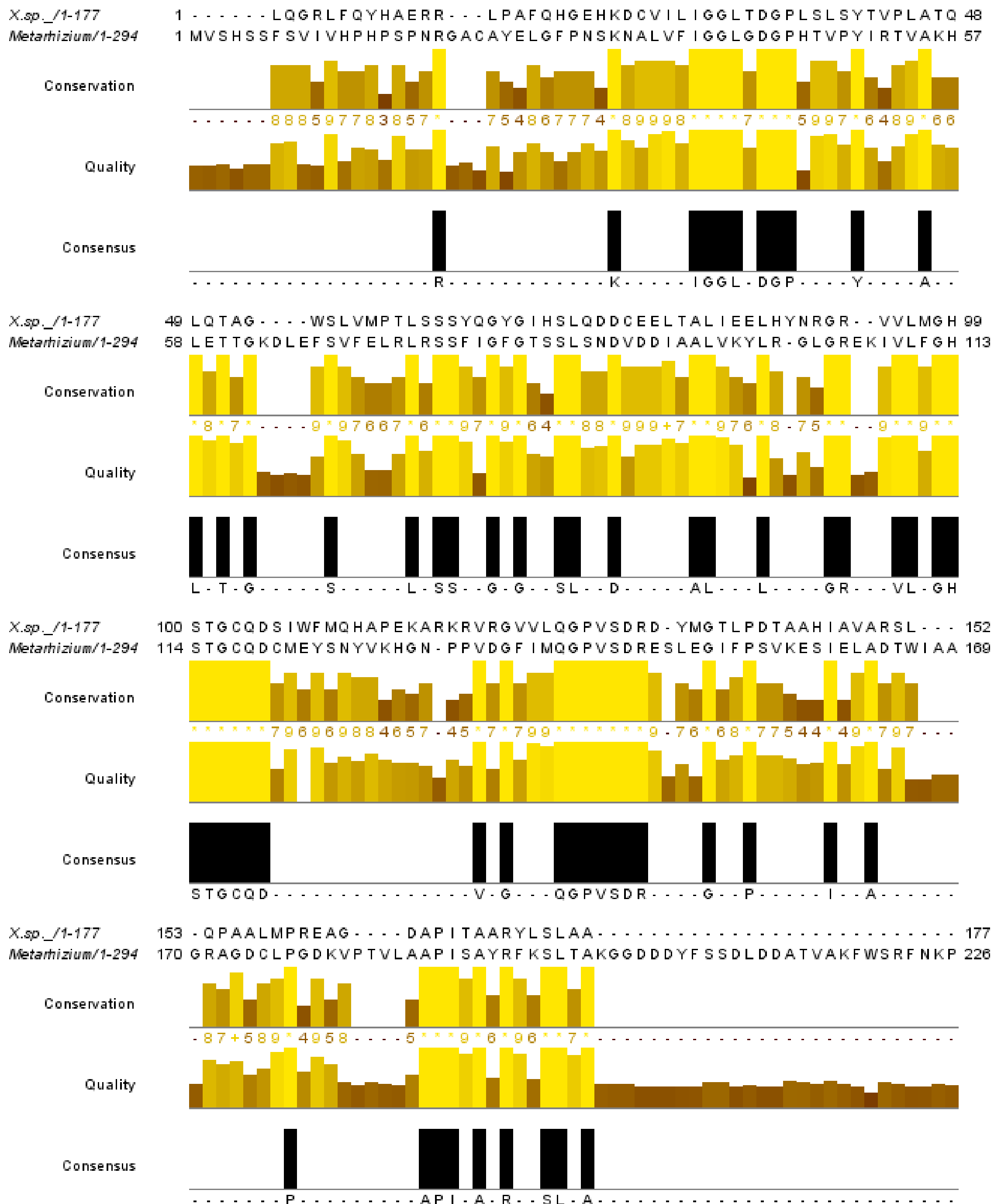

Fig. 10: Alignment of *H. coloradii* peptide with an esterase/lipase (EFY91335) from *Metarhizium acridum* (31% identity).

Several enzymes were found that are involved in the initial steps of lipid synthesis. Several fatty acid desaturases and elongases were also found. These are shown listed in Table 6. In addition, several long-chain fatty acyl-CoA synthetases were found in the genome of *H. coloradii.*

NCBI accession	**Protein description**
CBN79823.1	Acetyl-coenzyme A synthetase [*Ectocarpus siliculosus*]
YP_628900.1	fatty acid desaturase family protein [*Myxococcus xanthus*]
CBJ29129.1	fatty acid desaturase [*Ectocarpus siliculosus*]
CBJ26568.1	Fatty acid elongase [*Ectocarpus siliculosus*]
YP_001831427.1	fatty acid desaturase [*Beijerinckia indica*]
YP_002361678.1	fatty acid desaturase [Methylocella silvestris]
CBJ49159.1	Fatty acid desaturase [*Ectocarpus siliculosus*]
CBN78890.1	Fatty acid elongase [*Ectocarpus siliculosus*]
XP_002508575.1	fatty acid desaturase [*Micromonas sp. RCC299*]
YP_003277402.1	long-chain-fatty-acid--CoA ligase [*Comamonas testosteroni CNB-2*]
P12276.5	Fatty acid synthase [*Gallus gallus*]
ADG36330.1	Δ-4 fatty acid desaturase [*Pavlova viridis*]
CBJ28321.1	cyclopropane-fatty-acyl-phospholipid synthase/ oxidoreductase

	[*Ectocarpus siliculosus*]
YP_143714.1	long-chain-fatty-acid--CoA ligase [*Thermus thermophillus HB8*]
CBJ26568.1	Fatty acid elongase [*Ectocarpus siliculosus*]
CBN76062.1	fatty acid-ACP thioesterase [*Ectocarpus siliculosus*]
CBJ26764.1	polyunsaturated fatty acids Δ-6-elongase [*Ectocarpus siliculosus*]
YP_001846474.1	long-chain fatty acid ABC transporter [Acinetobacter baumannii]
CBN74970.1	Δ-6 fatty acid desaturase [*Ectocarpus siliculosus*]
XP_001260605.1	bifunctional fatty acid transporter/acyl-CoA synthetase [*Neosartorya fischeri*]
ZP_05095815.1	Cyclopropane-fatty-acyl-phospholipid synthase [*marine gamma proteobacterium*]
CAD53323.1	Δ 5 fatty acid desaturase [*Phytophthora megasporidium*]
YP_001519835.1	long-chain-fatty-acid-CoA ligase, putative [*Acaryochloris marina*]
ZP_01221855.1	omega-3 polyunsaturated fatty acid synthase PfaA [*Photobacterium profundum*]
YP_002361678.1	fatty acid desaturase [*Methylocella silvestris*]

ZP_05000329.1	fatty acid desaturase [*Streptomyces sp. Mg1*]
CBJ31067.1	Fatty acid desaturase [*Ectocarpus siliculosus*]
ADG36330.1	Δ-4 fatty acid desaturase [*Pavlova viridis*]
YP_001474935.1	omega-3 polyunsaturated fatty acid synthase PfaB [*Shewanella sediminis*]
XP_002774616.1	Long-chain-fatty-acid--CoA ligase, putative [*Perkinsus marinus*]
XP_002508575.1	fatty acid desaturase [*Micromonas sp. RCC299*]
AAF70457.1	Δ-5 fatty acid desaturase [*Homo sapiens*]

Table 6: Enzymes involved in desaturation/elongation found with BLASTP from *H. coloradii* peptides.

EPA synthesis:

Every fatty acid synthase needed to make EPA (Fig.11) was found in *H. coloradii*. Matches of *H. coloradii* peptides with enzymes in this pathway include a Δ 9 desaturase: (pep34467/ZP_06711707.1) from *Streptomyces sp. e14*, a Δ 12 desaturase : (pep9713/AAR20443.1) from *Saprolegnia diclina*, a Δ 6 desaturase: (pep44208/CBN74970.1), from *Ectocarpus siliculosus*, a PUFA Δ-6-elongase: (pep84363/CBJ26764.1) from *Ectocarpus siliculosus*, a Δ 5 desaturase : (pep49169/CAD53323.1), from *Phytophthora megasperma*, and an

Omega-3 fatty acid desaturase: (pep71855/AFJ69342.1*)*from *Nannochloropsis gaditana.* Alignments with close matches are shown in figures 12-15.

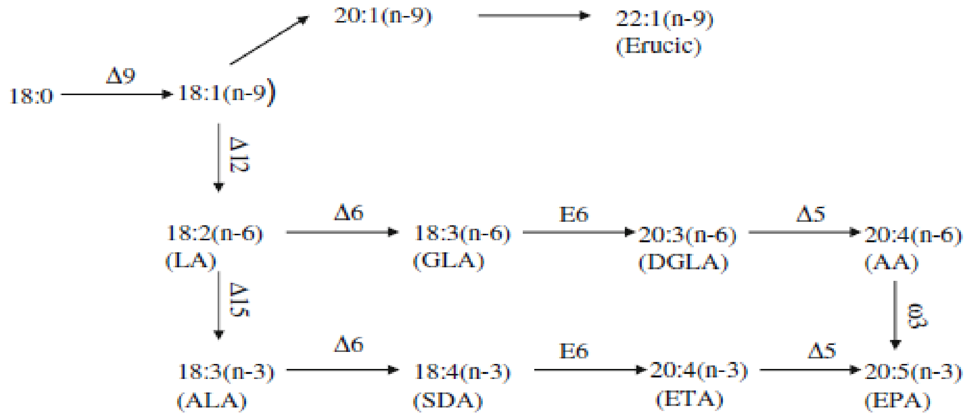

Fig. 11: Biosynthetic pathways of erucic acid, arachidonic acid (AA) and eicosapentaenoic acid (EPA). LA linoleic acid, ALA alpha-linoleic acid, GLA gamma-linolenic acid, SDA stearidonic acid, DGLA dihomo- gamma-linolenic acid, ETA eicosatetraenoic acid, AA arachidonic acid, EPA eicosapentaenoic acid.

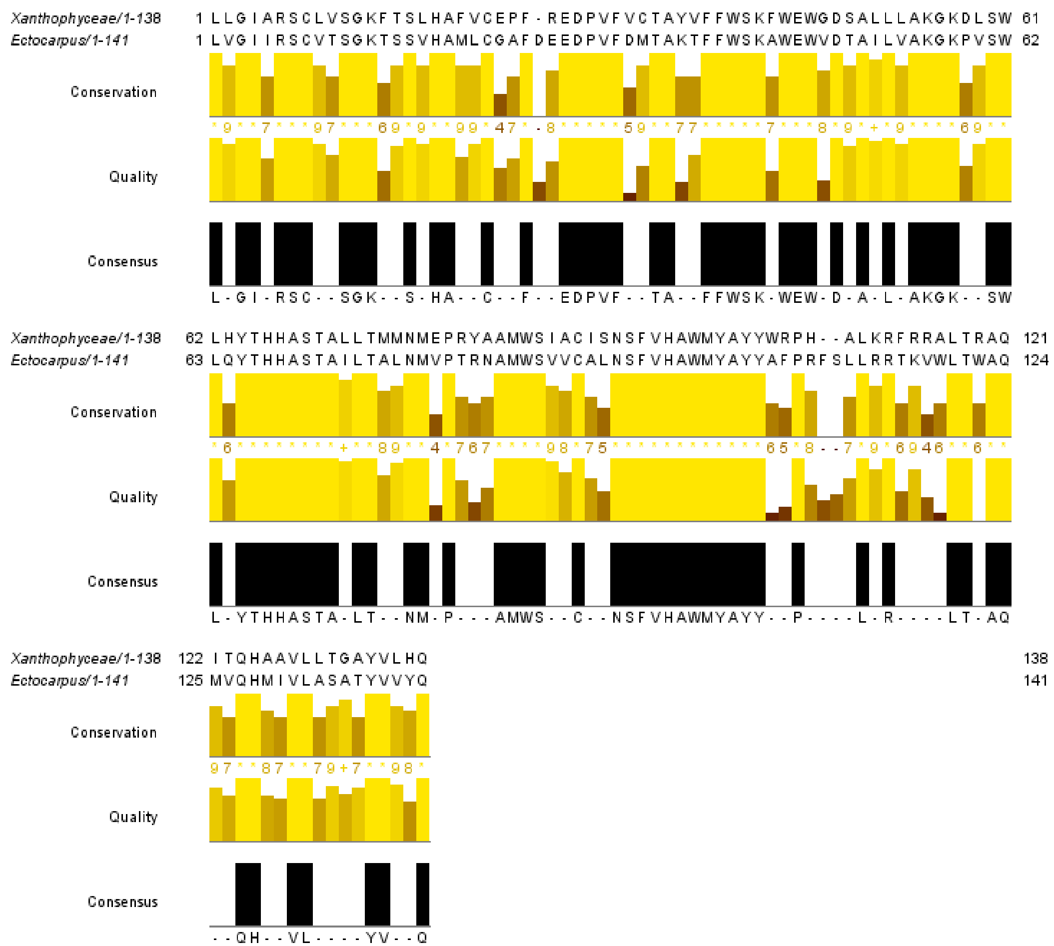

Fig. 12: Alignment of an *H. coloradii* peptide (pep84363) with a Δ-6-elongase from *Ectocarpus siliculosus* (CBJ26764.1) (58% identity).

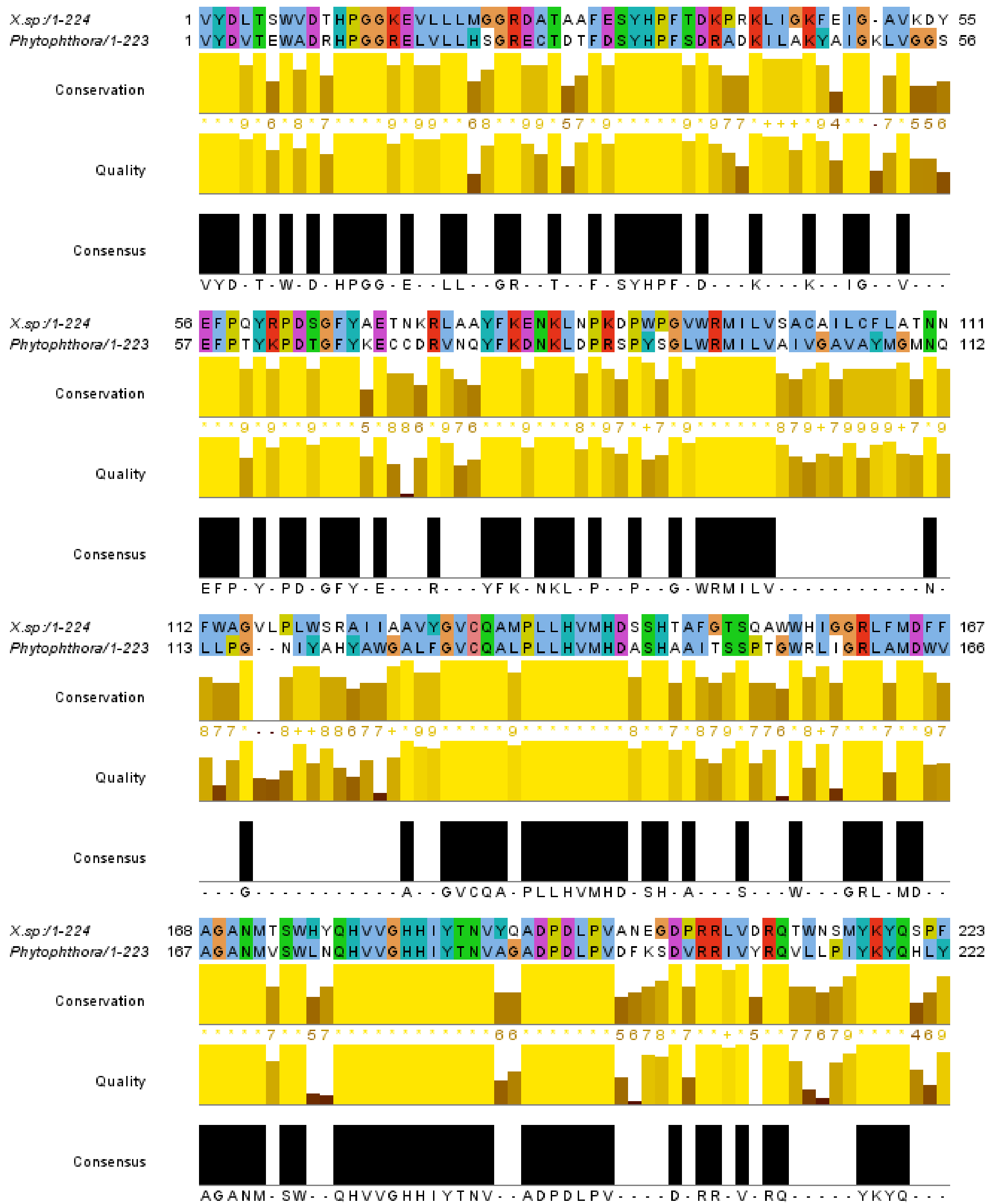

Fig. 13: Alignment of *H. coloradii* peptide (pep49169) with a Δ 5 fatty acid desaturase (Phytophthora, CAD53323.1) (51% identity).

Fig. 14: Alignment of *H. coloradii* peptide (pep71855) with a Δ-12 desaturase (CBJ31067.1) from *E. siliculosus* (60% identity).

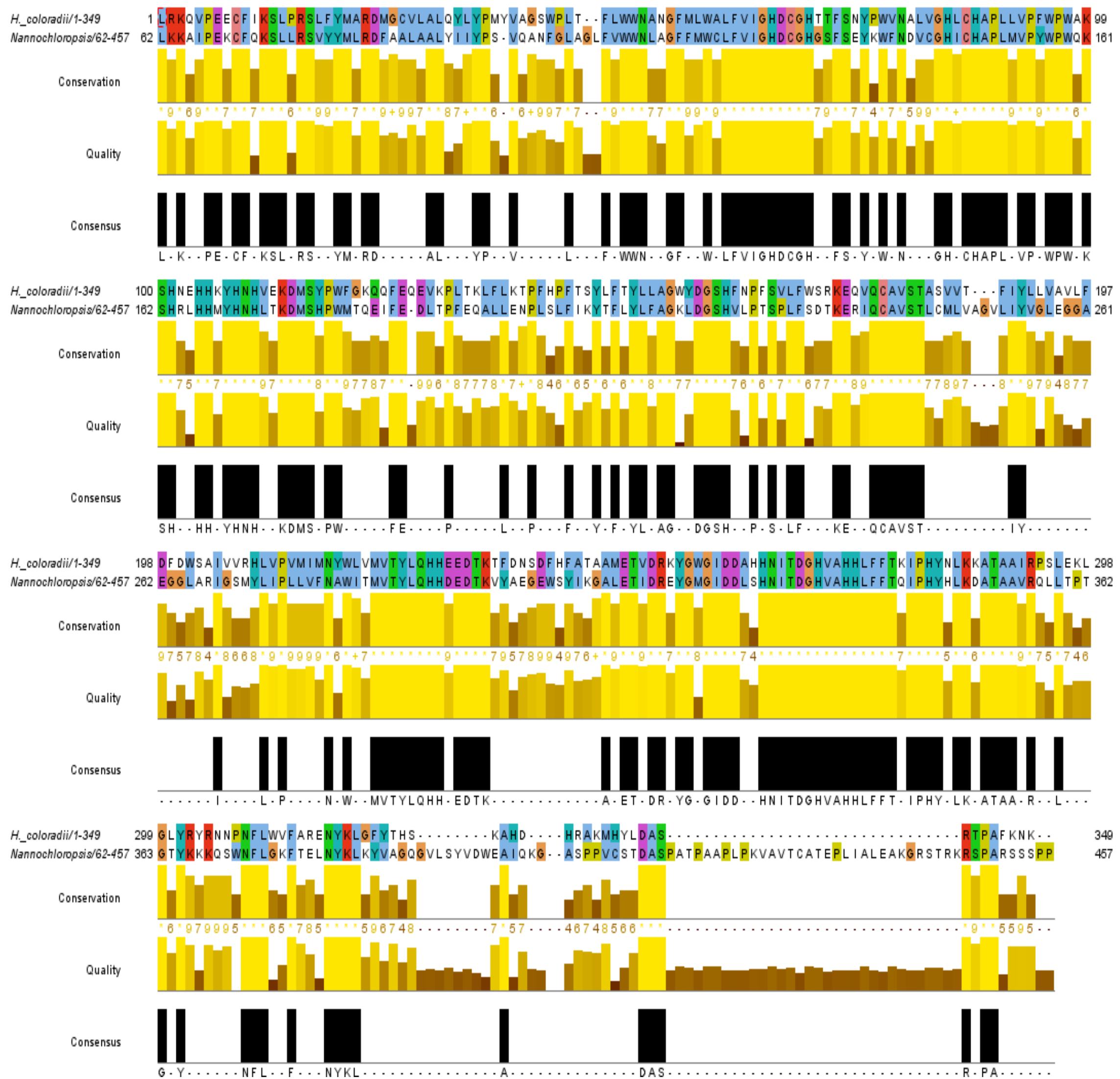

Fig. 15: **Alignment of H. coloradii peptide (pep71855) with an omega-3 desaturase from Nannochloropsis gaditana (AFJ69342.1) (44% identity).**

Virus Proteins

H. coloradii had 75/240 unique peptide-coding regions found with homology to EsV-1 proteins. EsV-1 is a lysogenic dsDNA virus *E. siliculosis* belonging to the super family of nucleocytoplasmic large DNA viruses (NCLDV) (Cock *et al.* 2010). Also found were 19 unique peptides from the *Feldmannia irregularis* virus and: 4 unique peptides from the Marseillevirus.df

Beta-Tubulin

H. coloradii was found to have a phenylalanine to tyrosine polymorphism in two copies of beta tubulin that differ from *E. siliculosus (*Fig. 16)*.* This polymorphism is the only nucleotide change between the *H. coloradii* and *E.siliculosus* beta-tubulin genes.

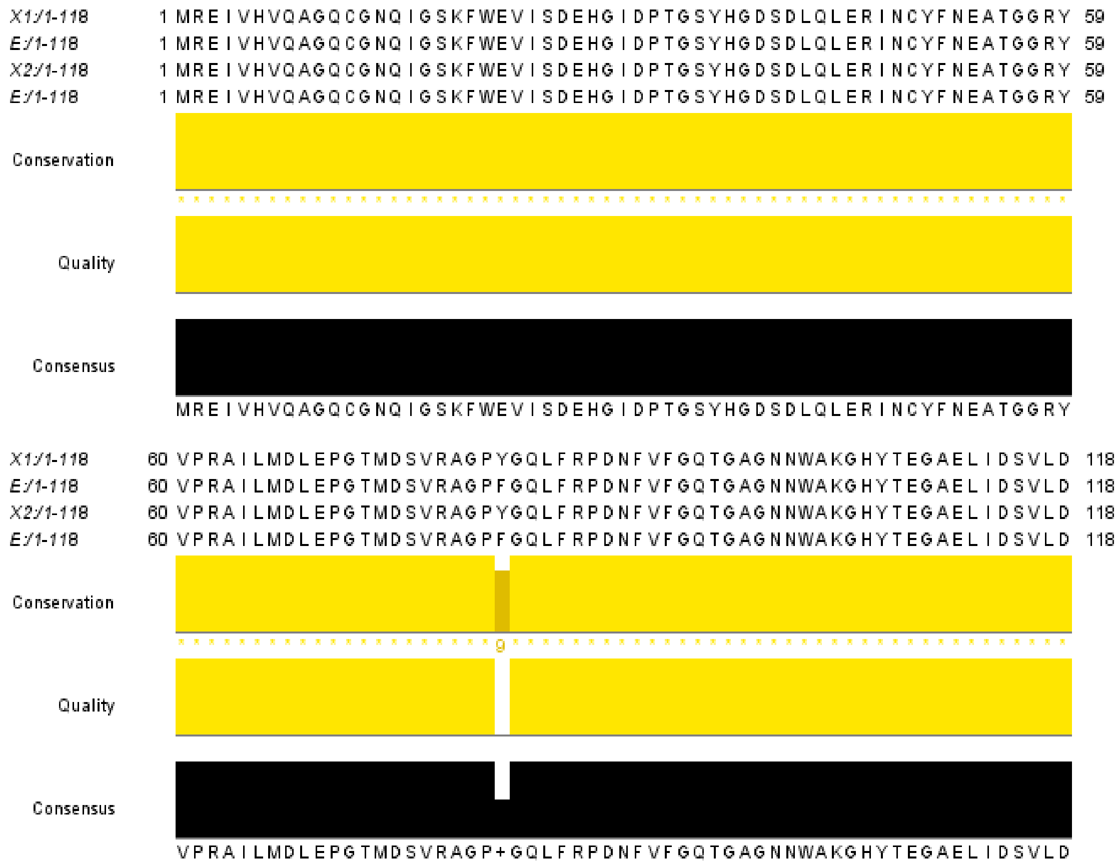

Figure 16: Alignment of beta-tubulin from *H. coloradii* and *E. siliculosus.*

DISCUSSION

Cold tolerance:

Many cold tolerant organisms have lower GC content to help them replicate and utilize DNA sequences (Casana and Gullati 2011). *H. coloradii* had a GC content of 48.69%, which is much higher than its close Mesophillic relative *E. siliculosis* with its GC content of 30.7% (Table 1). Therefore, *H.*

coloradii must have unique cellular mechanisms that help it adapt to cold climates, since it grows well at 4ºC.

H. coloradii was found to have 3 AFPs, which are essential for the survival of many organisms in cold climates. AFPs reduce the harm caused by the crystallization of water to cells (Carvajal-Rondanelli *et al.* 2010). Normally when ice expands it pushes proteins out of its way and away from its expanding front. AFPs are different in that they adsorb to the surface of the expanding ice and bind it (Carvajal-Rondanelli *et al.* 2010). An array of threonine residues within certain AFPs are known to aid proper binding to ice (Davies 2002), for example. In this case water molecules that are to become ice must bind in-between amino acids. The expansion of ice through narrow channels rather than as a uniform front is thermodynamically unfavorable. Thus the expansion of the intracellular ice is retarded or completely stopped by the AFPs (Wilson *et al.* 1993). Algae AFPs have been implicated in changing the structure of sea ice on a macroscopic level (Raymond 2011).

It is unknown why the AFPs from *H. coloradii* would match AFP from such a divergent group of organisms (figs. 3-5). While relatives of *H. coloradii* such as *E. siliculosus* may have lost such AFP, a common ancestor may have passed certain AFPs down to *H. coloradii* and organisms like the cold-tolerant frogs and worms represented in figs. 3-5. Evolutionary convergence may also be cited as the cause of *H. coloradii's* putative AFPs and the matches found in NCBI, however this is doubtful because of the similarity of the peptide chains.

However, since in AFPs the peptide chain composition is tied tightly to its function, as in the threonine residue arrays, evolutionary convergence may well explain the homology between *H. coloradii's* putative AFP sequences and those found in the NCBI nucleotide database.

The single nucleotide polymorphism between *H. coloradii* and *E. siliculosus* is interesting because it is the only difference in a structural gene between a mesophilic and psychrophilic organism. Phenylalanine to tyrosine mutations in beta-tubulin have been shown to be involved in antibiotic resistance in helminthes (Niciura *et al.* 2012). Other polymorphisms in beta-tubulin confer cold resistance because they promote elasticity of the peptide chain (Chiappori *et al.* 2012)

Lipid metabolism:

With 505 genes found to potentially be involved in lipid metabolism in *H. coloradii,* this sequencing effort represents a major step forward in describing lipid metabolism in *H. coloradii.* All genes involved in the EPA synthesis pathway have BLAST hits in the *H. coloradii* genome, although these newly discovered genes remain to be experimentally verified.

Studying the enzymes involved in EPA synthesis may provide information useful for heterologous expression of these enzymes for EPA production in other

organisms, or even to increase the amount of EPA increased in *H. coloradii*. For example, genes encoding a Δ6 desaturase, Δ6 fatty acid elongase, and Δ5 desaturase from the alga, *Phaeodactylum tricornutum*, were co-expressed in Pichia pastoris to produce arachidonic acid (ARA; 20:4 Δ(5, 8, 11, 14)) and eicosapentaenoic acid (EPA; 20:5 Δ(5, 8, 11, 14, 17)). In these studies each of these genes was introduced into *P. tricornutum* by transformation. The resulting transgenic *P. tricornutum* could produce up to 4x more EPA than the wild type *P. tricornutum*. End EPA levels were up to 0.1% EPA in transgenic *P. tricornutum* containing double copies of the Δ6 desaturase, Δ6 fatty acid elongase, and Δ5 desaturases while wild type *P. tricornutum* could produce up to 0.05% EPA of total fatty acids (Li *et al.* 2009).

While *P. tricornutum* is not widely used in the biotechnology industry, *Saccharomyces cerevisiae* is frequently used for production of a number of valuable biocompounds in addition to its wide use in food production (Keasling 2010, Yu *et al.* 2011). Thus, *S. cerevisiae* is an attractive target for trans genes leading to the production of EPA. Transformation of *S. cerevisiae* with heterologous Δ5 desaturases from the ciliate protozoan *Paramecium tetraurelia* and from the microalgae *Ostreococcus tauri* and *Ostreococcus lucimarinus allowed S. cerevisiae* to produce EPA using glucose as the sole carbon source (Tavares *et al.* 2011). An experiment could be conceived wherein *S. cerevisiae* is transformed with an *H. coloradii* Δ5 desaturase.

The breakdown of tryglycerides, including EPA, is catalyzed by lipases. Lipases act on carboxylic esters on glycerol-esterified fatty acids to free the fatty acids from the glycerol backbone (EC.3.1.1.-). The catalytic center contains three residues acting as acatalytic triad: a serine, a glutamate or aspartate and a histidine. These catalytic residues are responsible for the nucleophilic attack on the carbonyl carbon atom of the ester bond. Lipase reactions are critical for tryglyceride breakdown and are possibly responsible for the drop in certain fatty acids seen in *H. coloradii* upon change of environmental conditions.

In addition to lipase reactions, various transferase, desaturase, and elongase reactions are responsible for changing one fatty acid into another as the organism requires. An acetyl-CoA synthetase was found that provides an initial precursor, acetyl-CoA, for fatty acid production. Acetyl-coA synthetase catalyzes the reaction between ATP and acetate to form acetyl-CoA (KEGG). Acetyl-CoA is used to initiate and elongate fatty acid chains as well as provide precursors to a number of other energy-producing molecules (Starai *et al.* 2004).

Several long-chain fatty acyl-CoA synthetases were found in the genome of *H. coloradii* (Table 6). These enzymes perform important regulatory functions in cellular homeostasis, particularly in lipid metabolism (Kuar *et al.* 2011). A fatty-acyl CoA synthetase (EC 6.2.1.*x*) allows an otherwise non-reactive fatty acid to participate in a myriad of metabolic pathways. Fatty-acyl CoA synthetases in their differenct forms can catalyze reactions with short (2-3 carbons), medium (4-12 carbons), or long (>12 carbons) –chain fatty acid

substrates (Watkins 2007). Long-chain fatty acyl-CoA synthetases have been shown to be bi-functional enzymes involved in fatty acid transformation as well as transport, and they have several roles in the maintanaince of human health (Watkins 2007).

Virus Proteins

An adequate number of *EsV-1* proteins were found in the *H. coloradii* genome to conclude that either 1) *EsV-1* infected a common ancestor of yellow-green and brown algae or that 2) *EsV-1* has broader host specificity that previously thought and can also infect yellow-green algae. Since our study only analyzed translated peptide fragments, more sequencing will need to be done to address these possibilities.

Conclusion

We present here a draft version of *H. coloradii's* putative proteome, analyze its genes' functions and compare the organism to its closest relatives. The discovered genes offer doorways to several research venues to more specifically study *H. coloradii*. Overall, *H. coloradii* appears to be an interesting organism with a diverse collection of genes contributing to its unique lipid accumulation and cold-tolerance phenotypes.

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