Carnivory submerged: aspects of the ecology and ecophysiology of the aquatic *Utricularia stellaris* L. fil. (Lentibulariaceae) in South Africa

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Abstract

The trapping structures produced by aquatic species of *Utricularia* have traditionally been interpreted to function as adaptations to capture and break down zooplankton prey, as in other carnivorous plants, to overcome nutrient limitations. However, an increasing number of studies have found that these plants may also rely on benefits derived from living mutualistic microbial communities contained within traps. This study documents aspects of the environmental, growth and physiological characteristics of *U. stellaris* to inform and to form a basis for future investigation into the plant-microbe interaction.

The environmental conditions in which *U. stellaris* grows were documented to identify potential adverse conditions plants are subject to in situ, from which nutrient limitation was identified as a primary limitation. Plant growth and trapping structures were then assessed to identify possible adaptations of plants to overcome these limitations. The production of trapping structures likely constitutes an adaptive trait, with 30% of total biomass per node allocated to the production of these structures. Based on their capture function, traps may aid plants based on their contents, possibly supplementing plants with nutrients.

Although assessments of the habitats of *U. stellaris* indicate that dissolved CO_2 concentrations in the ambient water are high, CO_2 may still be limiting to the photosynthetic rates of these plants due to viscous water resisting the diffusion of CO_2. The primary site of photosynthesis in *U. stellaris* is leaves and trap tissue's contribution to photosynthetic output is negligible. *U. stellaris* plants are subject to CO_2 limitations in natural pond conditions, making the substantial allocation of resources to non-photosynthetic trapping tissue even more costly. Therefore, benefits gained from trapping structures are likely to be derived from trap contents; having ruled out the possibility that the trap tissue itself is photosynthetic.

Trap contents of *U. stellaris* were assessed. The proportion of traps containing living microbial communities greatly exceeded the proportion containing zooplankton prey. In addition, these communities were found to be diverse, stable, and self-sustaining. These results suggest that trapping structures may be beneficial for both the carnivorous capture of prey and the housing of living microbial communities. These results indicate the plant-microbe interaction in *U. stellaris* warrants further study.

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Chapter 1: General Introduction

Nutrient limitation in plants

A plant's survival under certain environmental conditions depends on its ability to evolve adaptations to overcome the stressors prevalent in its environment. One stressor that is a significant determinant of plant productivity is nutrient limitation.

Mineral nutrition in sufficient quantities is essential to the continued functioning of plant metabolism and, therefore, plant survival. Nitrogen and phosphorus are two macronutrients required in comparatively large amounts and are often the primary limiting nutrients determining plant growth (Aerts and Chapin, 1999). Nitrogen is the element required in the largest quantity and is central to the formation of proteins, nucleic acids, chlorophyll, co-enzymes, and other metabolites (Hawkesford et al., 2012). Phosphorus is another essential element, forming DNA and RNA and being vital to energy metabolism and transfer of cells as it is a component of ATP and NADPH (Hawkesford et al., 2012).

Nitrogen and phosphorus are differentially available to plants due to the relative availability of their sources (Tilman, 1986; Vitousek and Howarth, 1991), anthropogenic influences (Aerts et al., 1992), and the different ways these nutrients are cycled (Aerts and Chapin, 1999; Howarth, 1988) and, due to this, will be available to different extents in different environments.

Plant strategies to overcome nutrient limitation

The ability of a plant to mitigate nutrient limitation is a crucial determinant of its survival and persistence. To survive, plants utilise adaptations that increase the efficiency with which obtained nutrients are used, enhance the acquisition of already present nutrients or increase nutrient availability in itself (George et al., 2012).

Plants can increase the efficiency with which obtained nutrients are utilised by altering within-plant nutrient allocation, ensuring efficient transport of nutrients within a plant (Brown et al., 1967; Caradus and Snaydon, 1987), minimising senescence-driven nutrient loss (Aerts, 1995) and reallocating and reabsorbing nutrients from senescing biomass when senescence does occur (Uauy et al., 2006). Secondly, plants can enhance the uptake of nutrients that are already available. This uptake can be improved through specific root

morphologies and by developing high-affinity transporters at the site of uptake (Bucher, 2007; Lynch, 2005). In addition, plants can form a symbiosis with certain microorganisms, including mycorrhiza, which allows plants to acquire nutrients from areas outside of a plant's rhizosphere and increase a plant's ability to take up poorly mobile nutrients (Smith et al., 1992).

Lastly, plants can actively increase the available nutrients in their environment. Some plants can achieve this by exuding enzymes into the surrounding soil (McLachlan, 1980), exuding compounds supporting a nutrient-liberating soil microbial community (Clarholm, 1985; Martin et al., 1989) or utilising nitrogen sources fixed from atmospheric N_2 by supporting nitrogen-fixing symbionts (Soltis et al., 1995). In low-nutrient environments, specific subsets of plants have even evolved strategies that do not rely directly on nutrient availability in the abiotic environment altogether. These plants can obtain essential elements from the tissues of other living organisms through botanical carnivory, carnivorous plants being able to trap, digest and absorb nutrients from animal prey.

Botanical carnivory as an adaptive strategy to overcome environmental nutrient limitation

The carnivorous syndrome has arisen independently at least ten times throughout the angiosperms and is known to occur in at least 800 extant plant species (Ellison and Gotelli, 2001; Fleischmann et al., 2018). This distinct life strategy has evolved to supplement plant nutrient uptake in nutrient-poor, sunny, and at least seasonally wet locations (Benzinq, 1987; Givnish et al., 1984). These environmental niches are unsuitable for most other plants without adaptations to overcome nutrient limitations due to the negative impacts of nutrient deprivation on plant productivity and competitiveness. Therefore, carnivorous plants are suited to open habitats in various climatic zones where macronutrients are scarce (Ellison and Gotelli, 2001; Givnish et al., 1984; Juniper et al., 1989). The scarcity of available nutrients in the environments of these plants is countered through the trapping and digestion of prey, allowing these plants to obtain, primarily, phosphorus (Ellison, 2006; Stewart Jr and Nilsen, 1993; Wakefield et al., 2005) but also nitrogen, as well as less frequently targeted sulphur, potassium and magnesium (Ellison and Gotelli, 2001, 2009).

Surprisingly, the definition of a carnivorous plant lacked standardisation until Givnish et al. proposed criteria only in 1984, a definition that has formed an essential cornerstone to studies

of carnivorous plants following its publication. Recently, these criteria have been updated and refined (Ellison et al., 2018). For a plant to be classed as carnivorous, it needs to possess five specific traits: the ability to capture prey in specialised traps, the ability to kill the captured prey, digest this prey with a resultant absorption of nutrients and use these absorbed metabolites for growth and development (Ellison et al., 2018). Prey digestion as a precursor to nutrient absorption is sometimes achieved using digestive commensal organisms. But despite the indirect nature of this digestive process, these plants are still widely considered carnivorous (Król et al., 2012).

Diversity in trapping mechanisms

A distinct adaptation for carnivory in plants is the production of specialised trapping structures, the evolution of such structures having occurred independently in multiple plant lineages. The more than 800 recognised species of carnivorous plants occur across 19 genera, 12 families and five orders (Fleischmann et al., 2018). Throughout these plant orders, there is a notable degree of convergence amongst carnivorous taxa in terms of the morphology and physiology of trapping mechanisms (Ellison and Gotelli, 2009).

There are five types of traps recognised (Fleischmann et al., 2018), of which one of the most common is the flypaper trap, where specially evolved leaf glands exude a viscous, sticky mucilage to which organisms become stuck, expire and are subsequently digested. Flypaper traps can be either active or passive, passive flypaper traps including those produced by genera such as *Byblis*, *Drosophyllum* and most species of *Pinguicula* (Bauer et al., 2018; Król et al., 2012). As in other passive traps, no motion is involved in the trapping process. The flypaper traps produced by *Drosera* and some *Pinguicula* species are considered to be active traps, with either the stalked secretory glands or the leaf surface itself bending around a prey organism when it becomes stuck to the mucilage (Gibson and Waller, 2009; Legendre, 2000; Poppinga et al., 2018). In addition to being a relatively common strategy, the use of flypaper traps is also considered comparatively simple as opposed to more complex traps which include increasingly elaborate or specialised trapping mechanisms. These more complex mechanisms often involve active prey capture, increasing trapping predictability and the resultant capture frequency (Ellison and Gotelli, 2001).

Another commonly employed strategy, albeit entirely passive, is pitfall traps. One form of pitfall trap is used by the species of the monocotyledonous genera of carnivorous plants,

Brocchinia, *Catopsis* and *Paepalanthus* (Fleischmann et al., 2018; Król et al., 2012). These monocots are able to hold a basin of water within the central cavity formed by their rosettes of leaves into which prey organisms fall, are drowned and become a nutrient source for the plant. More specialised pitfall traps have evolved in five genera of dicotyledonous plants (Fleischmann et al., 2018; Król et al., 2012), the traps of these plants consisting of fluid-filled hollows in a central basin formed by tubular leaves. Prey organisms that move to the opening of the pitfall, on a rim that is often slick with the secretions of nectaries and trichomes (Bohn and Federle, 2004; Gorb and Gorb, 2006), tend to lose traction and fall into the vat of fluid below. They are barred from escaping due to preventative structures just below the rim, ranging from epicuticular waxes to cuticular folds (Poppinga et al., 2010). Trapped in the digestive zone, organisms drown and are subsequently digested.

Morphological trapping adaptations specialise further, using snap-trapping and suction-trapping mechanisms. The snapping-traps of *Dionaea* and *Aldrovanda* are bi-lobed, and the lobes snap together when the multicellular trigger hairs are stimulated by prey organisms (Hodick and Sievers, 1989), using secreted mucilage to create a wholly sealed trap environment during digestion (Juniper et al., 1989). As in the Droseraceae, the family Lentibulariaceae also contains carnivorous genera with more complex trapping mechanisms. The Lentibulariaceae is a diverse family comprising at least half of all described carnivorous plant species (Ellison and Gotelli, 2009) and includes the carnivorous genera *Pinguicula*, *Genlisea* and *Utricularia* (Ellison and Gotelli, 2009). *Pinguicula* is a genus of roughly 80 species, all of which are characterised as having a flypaper trap mechanism of prey capture. The genera *Genlisea* and *Utricularia* are characterised by more complex traps. *Genlisea* are unique in their use of passive yet complex eel traps for the capture of prey while *Utricularia*, utilise an equally unique suction-trapping strategy.

The genus *Utricularia*

Utricularia is the most species-rich genus of carnivorous plants, having undergone substantial diversification since its likely origin in South America (Silva et al., 2018). *Utricularia*, all being carnivorous, rootless herbs (Taylor, 1989) with over 230 species, make up 42% of all carnivorous plants, the greatest number of *Utricularia* species being found in the neotropics (Juniper et al., 1989). *Utricularia* are characterised by having very little

differentiation between stems, shoots and leaves, such a relaxed morphology often seen in plants adapted to aquatic habitats (Ellison and Gotelli, 2009).

The suction-traps produced by *Utricularia* are unique to the genus and are the fastest moving carnivorous plant traps (Poppinga et al., 2018). These traps are small, hollow, water-filled bladders derived from modified leaves which range in size from 0.2 mm to 12 mm long depending on the species (Płachno and Muravnik, 2018), with a hermetically-sealed trap door and delicate trigger hairs situated near this door. These traps are able to actively pump water out of the inner trap environment resulting in a negative pressure environment within the trap (Poppinga et al., 2018; Singh et al., 2011) which causes the flexible trap walls to be drawn inwards. The trap door of the utricle opens when the trigger hairs are disturbed by prey (Sydenham and Findlay, 1973) or when critical negative pressure within the trap is reached resulting in a spontaneous firing event (Adamec, 2011a; Poppinga et al., 2018; Vincent et al., 2011). The trap door opens resulting in a sudden release of stored elastic energy as the built-up pressure is released (Singh et al., 2011). The elastic utricle walls move outwards, pulling the water surrounding the trap door into the utricle, along with any organisms or detritus present in this water. The entire release and reclosing of the trap door takes only approximately 2.5 milliseconds, preventing the escape of organisms captured and minimising the outflow of nutrient rich water (Poppinga et al., 2018). Following a firing event, negative pressure is created within traps once again through the pumping out of water. A complete trap reset takes at least 15 minutes (Poppinga et al., 2018), but the resetting and subsequent firing rate depends on the size of the traps of different species (Adamec, 2011b).

The genus *Utricularia* consists of species with terrestrial, aquatic and facultatively epiphytic growth forms (Reut et al., 2021). Terrestrial species, terrestrial *Utricularia* being the ancestral state of the genus (Jobson et al., 2018), constitute the largest number of species. The growth form with the least number of species are the epiphytic *Utricularia*, exceeded in number by the aquatic growth form, with 27% of total *Utricularia* species being free-floating or affixed aquatics (Taylor, 1989). The submerged aquatic growth form is relatively uncommon throughout carnivorous genera, with aquatic carnivorous plants making up only 10% of all carnivorous species and having arisen in only two genera (Taylor, 1989).

Adaptations of aquatic *Utricularia* for a low nutrient environment

Aquatic *Utricularia* species characteristically inhabit water bodies with low nutrient availability. However, despite these limitations, plants are able to maintain a high level of productivity, as reflected by their rapid growth rates (Friday, 1989). This counter-intuitive productivity suggests that plants have developed some adaptation to overcome nutrient limitations in these habitats. One such adaptation that has been documented is the high nutrient re-utilisation efficiency from senescent shoots in aquatic *Utricularia*, with a maximum of 57% of nitrogen and a maximum of 77% of phosphorus reutilisation having been recorded in *U. australis* (Adamec, 2008). Another adaptation to their low nutrient environment is the production of trapping structures. Generally, there is a substantial biomass investment in the production of traps in aquatic *Utricularia* species (Richards, 2001), which indicates that these structures likely confer some benefit to the plant. In addition, *U. foliosa* displays a direct link between investment in trapping structures and the availability of nitrogen sources in the ambient water (Guisande et al., 2000; Manjarrés-Hernández et al., 2006), with a decrease in available nitrogen resulting in an increased number of and size of bladders. This correlation suggests that the biomass allocation to and production of trapping structures results from a need to enhance nitrogen uptake. The highly specialised function of these traps (Juniper et al., 1989), allowing for the capture of organisms and detritus in the water column, suggests that trapping structures provide these benefits to plants based on what is captured and contained within the traps. The trapping structures of aquatic species of *Utricularia* have traditionally been interpreted to function as adaptations to capture and break down nutrient-yielding zooplankton prey.

As per the criteria of botanical carnivory (Ellison et al., 2018), plants need to be able to capture prey organisms in specialised traps. This is certainly a criterion met in aquatic *Utricularia* species effectively capturing zooplankton prey with their highly specialised suction traps. Although chemo-attractive strategies have been suggested as a means of enhancing prey capture (Luetzelburg, 1910), this means of enhancement has not been satisfactorily quantified (Guisande et al., 2004, 2000; Manjarrés-Hernández et al., 2006). However, prey capture rates are enhanced by the presence of appendages and bristles surrounding trap doors (Meyers and Strickler, 1979), the positioning of which leads prey organisms in a funnel-like fashion towards the trap door. Following capture, these prey organisms are subsequently killed, likely due to the anoxic conditions documented within traps (Adamec, 2007), larger zooplankton being unable to survive under oxygen

concentrations less than 1 μM. This expired zooplankton is then digested, a process that is likely facilitated by microbes within the trap environment of *Utricularia* (Sirová et al., 2003). The subsequent absorption of nutrients evidences the digestion of prey in the trap environment, with Friday and Quarmby (1994) having shown that *U. vulgaris* takes up over 80% of prey nitrogen. Therefore, this criterion is met in at least some species of aquatic *Utricularia*. The final criterion that needs to be met by a carnivorous plant is the utilisation of absorbed nutrients for plant growth and development (Ellison et al., 2018). Investigations into the growth responses of aquatic *Utricularia* species to prey capture have shown mixed and ambiguous results (Adamec et al., 2010; Englund and Harms, 2003; Knight and Frost, 1991), likely due to these responses being species-specific and being significantly impacted by environmental factors, such as light intensity (Englund and Harms, 2003) and dissolved CO_2 concentration (Adamec, 2008). It is also possible that, through the mineralisation of captured organic detritus by microbial food webs (Sirová et al., 2018a), the microbial communities living in traps provide plants with sufficient nutrients that plants are less sensitive to prey shortages than other carnivorous plants (Adamec et al., 2010). Regardless, the aforementioned factors have impacted attempts to conclusively quantify the effects of nutrient gain from trapping structures. Thus, drawing conclusions about the use of prey-derived nutrients for growth and development in aquatic *Utricularia* species would be premature at this time.

When zooplankton capture occurs, the carnivory criteria are met in aquatic *Utricularia*. However, studies have found that the zooplankton trapping rates are remarkably low, with the proportion of traps containing prey being less than 50% in *U. australis* and *U. vulgaris* (Alkhalaf et al., 2009) and less than 10% of the traps assessed in *U. purpurea* (Richards, 2001). The subsequent nitrogen and phosphorus uptake from that which is captured is also low (Richards, 2001), theoretically too low to justify a 25-50% biomass investment into trapping structures (Bern, 1997). This low capture rate is unsurprising, as aquatic *Utricularia* grow in nutrient-poor environments where zooplankton are often scarce (Bern, 1997). In contrast to low zooplankton trapping rates, studies documenting trap contents of aquatic *Utricularia* species have noted that almost all mature traps contain living communities of microorganisms. These communities consist primarily of bacteria, algae, protists and rotifers, as well as non-living, organic detritus (Richards, 2001). Even though these microorganisms are too small to trigger trap firing, they are likely aspirated into traps during spontaneous trap firing events (Adamec, 2011a). The ubiquitous presence of these living microbial

communities suggests that a benefit may be derived from these communities within traps. This would explain the heavy investment in traps despite low zooplankton trapping rates. This theory is supported by findings that indicate high levels of nutrients are available within traps that lack macroscopic zooplankton prey but are colonised solely by living microbial communities (Sirová et al., 2009). This suggests that living microbial communities, the only contents of these traps, can create an inner trap environment characterised by high levels of available nutrients.

Are aquatic *Utricularia* solely reliant on carnivory?

Benefits derived directly from a trap that has captured zooplankton prey can be considered as benefits derived from carnivory (Ellison et al., 2018), even if microbial communities facilitate the digestion of such prey. Facilitation of prey digestion by commensals is particularly important to carnivorous plants that cannot produce digestive enzymes and is prevalent within the genera producing pitfall traps (Clarke et al., 2009; Ellison and Farnsworth, 2005; Frank and O'Meara, 1984; Gebühr et al., 2006; Moran et al., 2003). Perhaps the definition of a digestive organ should be reevaluated to recognise digestive mutualisms, should they be obligate and host specific, as an adaptation to carnivory (Anderson and Midgley, 2003). Regardless, *Utricularia* produces specialised trapping structures able to capture prey, and these traps create conditions that result in the expiration of this prey (Adamec, 2007). Prey is subsequently digested, possibly via microbial mutualists, and nutrients are derived from dead zooplankton within the trapping environment (Friday and Quarmby, 1994). Despite a lack of quantitative evidence supporting such (Adamec et al., 2010), these nutrients may be used for growth and development. However, this requires further study. Therefore, traps which capture zooplankton prey are comfortably carnivorous (Ellison et al., 2018).

However, benefits derived from traps containing only living microbial communities fall outside the definition of carnivory. Organisms are captured, but this capture does not result in the subsequent death of these organisms (Richards, 2001). Even though microbes forming the trap community are likely to expire eventually, following the natural timeline of senescence, being captured is not the cause of this expiration. Specialised structures for capturing prey are present, but if they do not capture prey bound to expire, perhaps they can alternatively be

considered as adaptations for the housing and, potentially, "cultivation" of commensal microbial communities.

Aquatic habitats are characterised by unique environmental stressors (Ellison and Adamec, 2011). While the primary stressor impacting terrestrial carnivorous plants has traditionally been understood to be nutrient limitation, followed in importance by sunlight and water (Givnish et al., 2018, 1984), in aquatic habitats, the photosynthetic rates of submerged macrophytes are often limited by low levels of available dissolved CO_2 (Marion, 2008; Smith and Walker, 1980). In addition, aquatic habitats are particularly reliant on microbial communities for nutrient cycling (Hahn, 2006) and *Utricularia* traps, submerged in this "inoculum", are easily colonised by these communities, far more readily than traps of terrestrial carnivorous species. Aquatic macrophytes are secondarily aquatic, suggesting that *Utricularia* evolved from terrestrial ancestors and moved into this unique aquatic niche. In response to this unique environment, the roles of traps may have diversified to take on an additionally beneficial role, namely, microbial commensalism. Despite the potentially beneficial nature of microbial trap communities having been suggested (Richards, 2001), these benefits have not, thus far, been sufficiently demonstrated and remain largely speculative.

Therefore, an important beneficial interaction may exist between living microbial communities and *Utricularia* in prey-free traps, in addition to the predator-prey relationship known to exist (Richards, 2001; Sirová et al., 2009, 2003). However, despite this theory having gained theoretical support, the role of microbial communities in *Utricularia* traps remains unresolved (Płachno et al., 2012).

Study species: *Utricularia stellaris*

This study focuses on a widespread species of aquatic *Utricularia, U. stellaris,* whose distribution spans much of Africa, including Madagascar, and parts of Asia and Australia (Figure 1.1) (Taylor, 1989). These plants are free-floating freshwater herbs with a single main shoot, or stolon, forming the length of the plant (Figure 1.2). Stolons are terete and approximately 0.5-1.5 mm thick, tapering towards the distal growing tip of the plant. While growth occurs at this distal end, the proximal end of the stolon undergoes simultaneous senescence (Rutishauser, 1993), resulting in a conveyer-belt type growth that allows it to maintain an approximately constant length (Adamec, 2018; Friday, 1989).

Figure 1.1: Global distribution of *Utricularia stellaris*, adapted from the Global Biodiversity Information Facility (GBIF, 2024).

Leaves are produced at the nodes along the stolon, with internodes increasing in length as the stolon matures. Bundles of leaves arise in a distichous order along the flanks of the stolon (Rutishauser, 1993). Leaves are highly dissected and consist of a central axis from which numerous secondary and tertiary leaf segments grow (Taylor, 1989). The secondary leaf segments are pinnate, with each pinna dividing dichotomously at its base into multiple further segments. The traps of *U. stellaris* are positioned at the bases of these secondary leaf segments. The traps of *U. stellaris* function the same way as other species in the genus (Singh et al., 2011), using negative pressure suction traps, which are obliquely ovoid and stalked, with a lateral mouth. In *U. stellaris*, these structures bear two dorsal, branched setiform appendages and lateral setae (Rutishauser, 1993). The traps have a comparatively large volume relative to other aquatic *Utricularia* species, with an average volume of approximately 1.5 µl (Singh et al., 2011) and a rapid resetting rate compared to other aquatic *Utricularia* species assessed (Adamec, 2011b; Singh et al., 2011).

The current literature on *Utricularia stellaris* consists of descriptive information (Taylor, 1989) and studies of trapping structure morphology and trap function (Adamec, 2011b; Kaur Cheema et al., 1992; Kurup et al., 2013; Singh et al., 2011). There is little information on the ecology of this species, with no investigation into the populations of this species on the African continent, constituting a distinct species gap within the widespread and unique genus *Utricularia*.

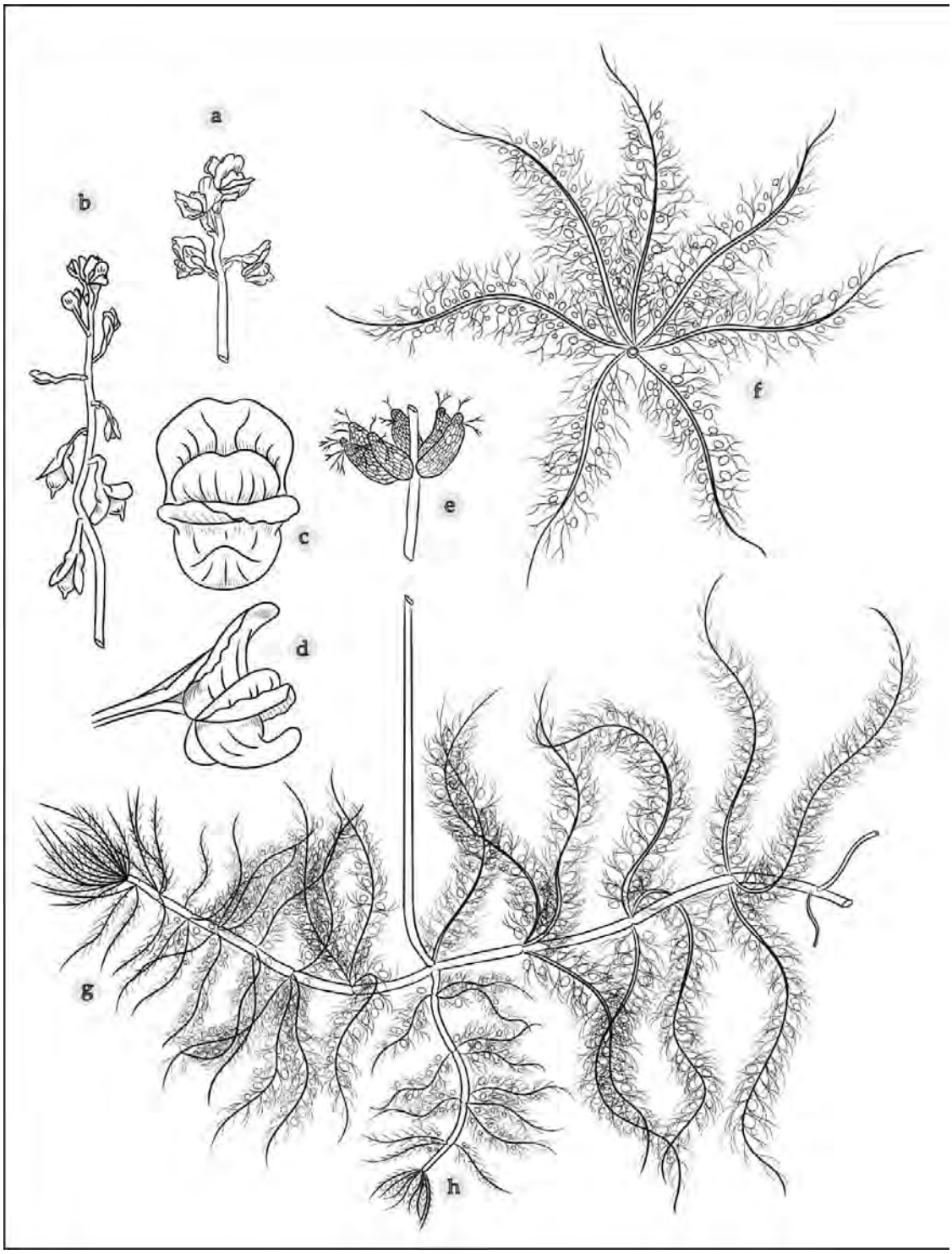

Figure 1.2: *U. stellaris*. (a) inflorescence in flower, x 1; (b) inflorescence setting seed, x 1 ; (c) flower, anterior view, x 4 ; (d) flower, lateral view, x 4 ; (e) whorl of floats at base of inflorescence, x 1 ; (f) transverse section through node 11, showing the orientation of leaves around the stolon, x 1 (g) first 11 nodes from the apical meristem, x 1; (h) side branch, allowing for clonal reproduction, x 1. Illustration by A. Marais.

Problem statement

Despite increasing studies, the role of microbial communities in the success of aquatic *Utricularia* species remains largely unresolved. It is possible that aquatic *Utricularia*, in adapting to the unique aquatic environment, has become reliant on microbial mutualism in addition to carnivory. Depending on the capture rate and resultant benefits, the proportion of nutrients derived from microbial communities may even exceed those provided through carnivory. Traps may, therefore, be important to plants not only as a means of prey capture but also for their ability to facilitate mutualism. However, understanding the function of trapping structures of aquatic *Utricularia* in their entirety requires resolution of the plant-microbial relationship.

Aims and hypotheses

This study aims to document aspects of the environmental, growth and physiological characteristics of *U. stellaris* to inform and to form a basis for future investigation into the plant-microbe interaction.

Plants evolve adaptive traits to overcome stressors in their environments. It is necessary to document the stressors in the aquatic habitats of *U. stellaris* to identify the factors driving adaptation in these plants. Therefore, in the second chapter of this thesis, I will assess the pond habitats in which this species grows to document potential environmental stressors. What is known about the habitats of carnivorous plants led to the hypothesis that nutrient availability in these habitats is likely low. To assess the validity of this hypothesis, the water nutrient status of these habitats will be assessed. In addition, the water chemistry of *U. stellaris* ponds, as well as diel fluctuations in dissolved gases, will be measured. If there are factors typically limiting to plants present in the pond environment of *U. stellaris*, then these plants may have evolved adaptations to survive under such conditions. This chapter will also assess whether plants exhibit adaptation to the documented environmental stressors. One hypothesised adaptive trait unique to *Utricularia*, the production of traps, will be described for this species. If there is a significant biomass allocation to trapping structures, these structures may provide some adaptive advantage to plants, potentially allowing them to survive despite being subject to the documented stressors.

The knowledge that CO_2 availability can often be limiting to photosynthetic rates in freshwater systems despite high ambient dissolved CO_2 concentrations led to the hypothesis that dissolved CO_2 concentrations may be limiting to the photosynthetic rates achievable by *U. stellaris*. The third chapter of this thesis more closely examines this possibility. This assessment will be completed by creating CO_2 response curves under controlled conditions and using these curves to model what photosynthetic rates of *U. stellaris* would theoretically be under stagnant pond conditions. If dissolved CO_2 concentrations are limiting, a stressor that is unique to carnivorous plants with a submerged aquatic growth form will have been documented. In addition, the photosynthetic capacity of trap tissue will be assessed. If trap tissue is a significant contributor to photosynthate production, then it is possible that one of the primary benefits of traps may simply be their ability to increase the net photosynthetic capacity of plants. However, if traps do not photosynthesise at a significant rate, then they are important for some other beneficial function, perhaps for benefits derived from what they contain.

Preliminary surveys of the contents of fresh *U. stellaris* traps indicated that traps containing zooplankton are scarce in comparison to those containing living microbial communities. This observation led to the hypothesis that microbial communities are ubiquitous throughout functional traps and that the proportion of traps containing living microbial communities exceeds that containing zooplankton prey. The fourth chapter of this thesis will document the trap contents of *U. stellaris* and provide an indication as to whether trap microbes are ubiquitous while traps containing prey are in the minority. Additionally, if the microbial community within traps is stable and self-sustaining, it is possible that these communities may be benefitting from being housed within traps. These results would indicate microbial communities may be benefitting plants and that the plant-microbe relationship requires further investigation in *U. stellaris*.

The results gathered here will aid in documenting this understudied system, clarify the environmental stressors that have driven adaptation, and assess the importance of traps as an adaptive strategy in *U. stellaris*. In addition, results will indicate whether the proportion of traps and the extent to which prey containing traps are in the minority and whether the microbial communities are ubiquitous enough to justify further investigation into the plant-microbe relationship.

Chapter 2: Adverse environmental conditions governing adaptation in the submerged aquatic plant *Utricularia stellaris* L. fil. (Lentibulariaceae)

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Introduction

Plants are sessile and, due to this constraint, cannot relocate in response to challenging environmental conditions. Due to this inability to escape adverse conditions in space, plants must adapt to ensure their continued survival, adaptations that are reflected in the resultant growth forms and physiological processes within plant tissues. Freshwater macrophytes are a group of plants generally characterised by a specific suite of adaptations gained in response to the unique selective pressures imposed by aquatic habitats (Bornette and Puijalon, 2011; Sculthorpe, 1967).

The temperature of the ambient water is one such unique factor. Unlike the air surrounding the surfaces of terrestrial plants, water has a buffering effect on the temperatures to which aquatic plants are exposed. The rates of processes such as photosynthesis in macrophytes are significantly impacted by water temperatures (Santamaría and van Vierssen, 1997), where maximum rates are only attainable when exposed to temperatures representing the optima of a particular species, with temperatures both too high and too low being limiting. Water temperatures also influence factors such as the concentrations of dissolved gases in this water (Pokorný and Květ, 2007), once again influencing biological processes such as photosynthesis. Not only does sunlight's heat influence biological activity but also the irradiance itself, being an essential determinant of a system's photosynthetic output and primary productivity. Water has an effect on the irradiance that is able to pass through the water column and reache a plant's surface, largely determined by the water's surface reflectance (Pokorný and Květ, 2007), refractory properties (Brusseau et al., 2019) and clarity (Brusseau et al., 2019; Sculthorpe, 1967). Water clarity is primarily determined by the suspension of solids in the water column. Suspension of solids is influenced by the movement of water in a system, the characteristics of the substrate lining a water body and the abundance of living microorganisms suspended in the water column (Brusseau et al., 2019),

photosynthetic microorganisms sometimes competing with macrophytes for light. Sediment characteristics and water motion, in addition to water clarity, also influence the likelihood of seed germination and anchorage of the resulting rooted macrophytes (Barrat-Segretain, 1996; Puijalon et al., 2005). Sufficient anchorage and robust rooting structures are particularly important in macrophytes reliant on nutrient uptake through roots (Barko and Smart, 1986), such as those in environments where the ambient water is nutrient-poor. Sediment nutrient availability depends on the sediment microbial community, which can liberate otherwise unavailable nutrients, influence the gaseous status of these sediments and determine potential toxicity due to microbial respiratory byproducts (Pezeshki, 2001).

The abundance of phytoplankton and resultant water turbidity is mainly determined by the nutrient status of water, with these free-floating organisms, just like free-floating macrophytes, solely relying on ambient water for nutrient gain and subsequent productivity. Not only do free-floating organisms impact the light available to macrophytes, but also sessile periphyton, which requires a substrate for attachment and survival (Pokorný and Björk, 2010), this substrate often being the surfaces of macrophytes. Submerged macrophytes also compete for light with other macrophytes, such as free-floating aquatics, which may shade the macrophytes at greater depths in the water column (Szabó et al., 2022). Thus, the nutrient status of a system impacts biota and biotic interactions, the most influential factors governing the photosynthetic output of submerged primary producers being the availability of carbon and the macronutrients nitrogen and phosphorus (Bornette and Puijalon, 2011). Aquatic plants take up phosphorus as phosphate (Thiébaut, 2008). However, nitrogen is available in more than one form, namely nitrate (NO_3^-) and the ionised, water-soluble form of ammonium, NH_4^- (Ghaly and Ramakrishnan, 2015). Phosphorus is frequently found to be the most limiting nutrient in freshwater systems (Schindler, 1974; Vollenweider and Kerekes, 1982), less prone to liberation from sediments than nitrogen sources. Due to the varying efficiency with which different organisms can take up these elements, limitations are often driven by biotic interactions, with phytoplankton populations being particularly efficient at nutrient stripping (Pedersen, 1994). Not only are nitrogen and phosphorus influential, but also the level of water mineralisation due to microelements and other dissolved solids, determining the salinity and, therefore, osmotic pressure of a system (Boyd, 2000a). If salinity is too high, it may result in a loss of water from the tissues of organisms, negatively impacting those organisms that lack adaptation to such conditions.

Freshwater systems are characterised by a unique set of conditions compared to terrestrial systems. Similarly, freshwater systems differ due to the variable levels of water turbulence characterising a system, spanning from lotic, flowing conditions to lentic, largely stagnant water bodies.

In lentic systems, the lack of water motion has numerous impacts on the factors noted above. Water turbulence is at a minimum which minimises the resuspension of the substrate and, therefore, has a minimal impact on light availability. This means that the primary factors limiting the light reaching submerged macrophytes is water depth and biota such as phytoplankton suspended in the water column, periphyton colonising plant surfaces and macrophytes higher in the water column shading those below. Free-floating macrophytes, granted sufficient nutrient availability, are particularly prevalent in lentic systems, as the lack of flow prevents the washing away of these unanchored plants, and the free-floating growth form allows them to remain in the area of high light availability at the water's surface.

Another condition prevalent in lentic systems is the frequent disequilibrium between concentrations of dissolved gases and the concentrations of these gases in the atmosphere (Smith and Walker, 1980). These differences arise due to both the slow gaseous diffusion rate through a medium as viscous as water (Marion, 2008) and the hugely active biotic component of these systems, driving diel fluctuations in dissolved O_2 and CO_2 based on the levels of photosynthesis and respiration (Keeley, 1998). These fluctuations result in varying degrees of O_2 and CO_2 availability to macrophytes dependent on the time of day, being particularly distinct in lentic systems where a lack of stirring and water mixing results in more extreme deficit gradients.

Lentic freshwater systems are characterised by a suite of conditions unique to these systems. These stressors have resulted in certain adaptations commonly seen in freshwater macrophytes that allow them to survive under such conditions.

One such morphological trait freshwater macrophytes employ in lentic conditions is adopting a free-floating growth form. Free-floating plants are able to remain at shallow depths with sufficient light availability regardless of changes in water depth. However, the prevalence of this growth form is dependent on the nutrient availability in the ambient water. The ambient water is the only source of nutrients for free-floating macrophytes, necessitating high nutrient

availability (Lacoul and Freedman, 2006). Ensuring nutrient uptake driven certain adaptations in these plants in the form of a thin cuticle (Reut et al., 2021), enhancing the direct uptake of dissolved nutrients through surfaces. Survival despite a thin cuticle is possible in aquatic habitats where mechanical damage is limited. In addition, these plants are often capable of rapid growth, which counters periphyton colonisation. Rapid growth allows aging, heavily colonised plant material to senesce and young uncolonized material to be produced, ensuring sufficient light exposure (Friday, 1989).

Other morphological specialisations thin and highly branched leaves. This leaf structure is advantageous in that it minimises self-shading while maximising exposure to sunlight and dissolved gases in the ambient water (Reut et al., 2021), supporting photosynthesis. Photosynthetic rates are further enhanced by chloroplasts being concentrated in epidermal cells. These cells can be characterised by numerous infoldings, increasing surface area and maximising the gaseous exchange possible between the water and these cells (Rascio et al., 1999). In addition, many aquatic plants have an internal network of aerenchyma throughout tissues that, in addition to maintaining buoyancy, allow for efficient movement of gases, both taken up and produced, throughout the plant (Rascio, 2002). Certain plants have also evolved physiological and biochemical strategies to ensure efficient gaseous exchange with the ambient water, such as carbon-concentrating mechanisms (CCMs). The most commonly employed CCM in freshwater macrophytes is the ability to utilise bicarbonate (HCO_3^-) as an inorganic carbon source for photosynthesis (Maberly and Gontero, 2017), this being the most commonly available inorganic carbon molecule in alkaline waters. Some freshwater macrophytes are even adapted to utilise a C_4-like carbon concentrating mechanism (Holaday and Bowes, 1980), while others use physiology similar CAM photosynthesis, Aquatic Acid Metabolism, as a means to take advantage of the elevated levels of respiratory CO_2 that becomes available at night (Suissa and Green, 2021).

One group of freshwater macrophytes well adapted to their aquatic habitat is the aquatic species of the genus *Utricularia*. These plants have been documented to mainly inhabit lentic freshwater bodies with low nutrient availability (Guisande et al., 2007; Juniper et al., 1989) and high concentrations of CO_2 in the ambient water (Adamec, 2008; Moeller, 1978). These plants exhibit adaptation to their aquatic habitat, and even within the single genus *Utricularia*, a range of increasing specialisation to an aquatic habitat has been documented

(Reut et al., 2021). Morphological adaptations include their narrow, thin leaves, minimising self-shading and enhancing the absorption of light and dissolved gases (Sculthorpe, 1967) and an extensive internal network of aerenchyma, enhancing buoyancy and gaseous exchange (Reut et al., 2021). These plants are also known to undergo rapid growth, effectively mitigating periphyton shading (Friday, 1989). Many of the aquatic species of *Utricularia* free-floating and have developed a reduced cuticle to enhance nutrient uptake and exposure to sunlight (Reut et al., 2021). The single case where a CCM was found within the genus is only induced under extreme conditions and is, therefore, of little ecological importance (Adamec, 2018, 2009).

Another adaptation that aids in the survival of aquatic members of the genus *Utricularia,* although the full benefit of these structures remains unresolved, is the production of hollow utricles on leaves. These structures can draw in, capture, and contain organisms and detritus from the surrounding water column (Juniper et al., 1989), traditionally interpreted to be solely beneficial for the carnivorous breakdown of prey. Like other adaptations specific to the aquatic habit exhibited within the genus, trapping structures may have taken on addition roles unique to aiding plants in surviving the unique aquatic habitat. However, this possibility, the ecology and ecological adaptations of one such species of aquatic *Utricularia*, *U. stellaris* has remained undocumented despite being a widespread and important component of the free-floating macroflora of African water bodies.

Aims and Hypotheses

Freshwater lentic habitats pose several challenges to submerged macrophytes, and, in turn, these plants have evolved adaptations to enhance competitive ability and survival under such constraints.

This study aims to document certain environmental conditions in which *U. stellaris* populations grow in the Eastern Cape, South Africa. This will allow for the identification of potential environmental stressors that are acting on these plants. What is known about the habitats of other aquatic *Utricularia* species has led to the hypotheses that the primary stressor in these habitats will be low nutrient availability and that these habitats will be characterised by high CO_2 availability in the ambient water.

Additionally, the growth form of the little-studied *U. stellaris* will be documented, focusing on trapping structures to identify possible strategies employed by *U. stellaris* to withstand the identified environmental stressors. Based on what is known about other aquatic *Utricularia* species, it is hypothesised that *U. stellaris* will exhibit morphological adaptation to the aquatic habitat, that trapping structures will constitute a substantial resource investment, and that traps will function as is expected, drawing in and being able to hold water surrounding traps.

Methods and Materials

Study sites

To assess the water conditions of the sites where *U. stellaris* grows, 15 sites were selected in the Eastern Cape of South Africa, from Wesley (Site 1, Appendix A) moving north up the coast to Kabakazi (Site 15, Appendix A), spanning a north-to-south distance of 140 km (Appendix B). The southernmost site represents the most southern known extant population of this species on the African continent.

The lentic ponds in which *U. stellaris* grow are small and shallow, not deeper than 3 m, several being pools of water accumulated in roadside depressions. Generally, water clarity is high and the plants can easily be seen through the water from above, with relatively high light penetration to the depth at which *U. stellaris* grows. The substrate of ponds is mostly fine and largely organic, releasing a substantial amount of accumulated gases when disturbed. An exception is site 7 / 8, consisting of a pool of water accumulated on a base of rock that has been anthropogenically cut away and exposed. Ponds are characterised by a relatively low level of emergent vegetation and most ponds are co-habited by the non-carnivorous *Nymphaea nouchali* and *Nymphoides thunbergiana* and some with *Wolffia* spp. Site 15 was the only site that was habitat to both *U. stellaris* and *Isoetes wormaldii*.

Water chemistry assessment

At each site, the pH, conductivity, and total dissolved solids (TDS) were recorded at five points throughout each pond using a SensoDirect 150 (Lovibond, Germany). In addition, a single water sample was collected per site in a 200 ml acid-washed bottle and placed in a cooler for later nutrient analysis. In the laboratory, samples were analysed for nitrate-nitrogen (NO_3^--N), nitrite-nitrogen (NO_2^--N), orthophosphate-phosphorus (PO_4-P) and ammonium-

nitrogen (NH_4^+-N). PO_4-P and NH_4^+-N were analysed using Spectroquant® concentration test kits. NO_2-N was analysed according to the American Public Health Association (APHA, 1992) on a Biotek microplate reader at 540 nm. Nitrate-nitrogen was analysed according to Ondrus (1996) using a microplate reader. Total inorganic nitrogen (N_t) and total inorganic phosphorus (P_t) concentration was calculated by summing the nitrogen components of NO_3^--N, NO_2^--N and NH_4^+-N and calculating the phosphorus component of PO_4-P based on the molar mass ratio of nitrogen and phosphorus to other elements in each nutrient (Odume et al., 2012). All nutrient analyses were conducted in triplicate, with three replicates of water from a single site being tested.

24-hour environmental fluxes

To elucidate how pond conditions fluctuate over 24 hours, dissolved carbon dioxide, dissolved oxygen (DO) and temperature were measured at a single site, site 11 (Appendix A), for 48 hours towards the end of summer, from the 3rd to the 5th of March 2023. For the duration of the measurements, there was no cloud cover. Site 11 is a small permanent pond supplied with water year-round via a spring below the pond. The pond is no deeper than 2 m and the substrate is fine and organic, releasing gases when disturbed. The water is relatively clear, being able to see *U. stellaris* individuals when viewed from above. This pond is lentic and well sheltered by surrounding trees, being largely undisturbed even on windy days. The primary plants inhabiting this site include *U. stellaris* and *Nymphaea nouchali* but, overall the site is not densely vegetated.

Every two hours for 48 hours, four readings of DO and temperature were taken throughout the pond using a SensoDirect 150 (Lovibond, Germany). From each point at which DO and temperature were taken, a 200 ml water sample was collected in a glass bottle, which was sealed promptly following collection. In situ, titration was used to determine the concentration of dissolved CO_2 in these water samples. The 200 ml water sample was titrated against a 0.0227 N sodium hydroxide solution using a phenolphthalein indicator until an endpoint was reached.

All titrations were conducted within a half-hour of sample collection to minimise changes in dissolved CO_2 in water samples over time (Pfeiffer et al., 2011).

Plant growth form

To describe the growth form of *Utricularia stellaris* and identify potential adaptive strategies for its aquatic habitat, plants were obtained for evaluation. Sampling was carried out on the 29th and 30th of April 2021 at three sites near Morgan Bay, Eastern Cape, South Africa (Appendix A, sites 1(a), 2(a) and 3(a)). Six whole plants were collected at each site. Because *Utricularia stellaris* grows in large numbers, the sampling was not detrimental to the populations. To collect whole plants, a plastic bag was submerged, and a single *U. stellaris* plant was floated into the submerged bag, which was subsequently sealed. Care was taken to ensure that the entire plant remained submerged to prevent the aspiration of air bubbles into traps. All samples were returned to the lab, stored in the dark at 8 °C and processed within four days following collection (Richards, 2001).

The internode length was measured for the entire plant, and the number of leaves growing from each node was counted. Leaf length was measured for one leaf at every fourth node along the stolon from the apical meristem. All measurements were taken using digital callipers under an Olympus SZH-ILLD stereomicroscope.

Pigment assessment

To document the Chlorophyll and Anthocyanin concentrations within young and mature structures of *U. stellaris,* plant material was collected from site 7 (Appendix A) on June 25th, 2023, and transported back to the laboratory for pigment analysis.

Chlorophyll *a*, chlorophyll *b,* and anthocyanin pigments were quantified and compared in young and mature trap, leaf and stolon material. Plant material from node 13 represented young plant material, while material collected from node 20 represented mature plant material. Five leaves were removed from node 13, and six lengths of stolon were collected, spanning from node 13 and working backwards towards the apical meristem. Approximately 100 large traps were removed from the excised leaves. All remaining traps were stripped off the leaves and discarded. The same collection of plant material was repeated for leaves at node 20, stolon material being collected from node 20 towards the senescing end of the plant. The wet weight of all samples was recorded prior to pigment extraction.

All extractions were conducted in a naturally dim laboratory where the fluorescent lights remained off. While awaiting quantification, all samples were covered in aluminium foil to minimise exposure to sunlight. Ice-cold analytical grade HPLC methanol (Minema, South

Africa) was used for all pigment extractions. The separated plant material was ground in a porcelain mortar and pestle in 1.5 ml methanol extractant, which was then vortexed and centrifuged for 3 minutes at 16,300 x g. 1 ml supernatant was transferred into fresh tubes. The pellet was resuspended in a new 1 ml methanol extractant, and the procedure was repeated. A total of 2 ml of combined supernatant was pipetted into a quartz cuvette. Chlorophyll *a* and *b* concentrations were determined by recording the absorbance at 652 nm and 665 nm using a Unico SQ-4802 double-beam spectrophotometer. Chlorophyll *a* and *b* concentrations were calculated according to the equations published for absolute methanol in Ritchie (2006).

To determine anthocyanin concentrations, an HCL and Milli-Q water solution was added to the methanol extraction in the cuvette (90:1:1) to degrade the chlorophyll in the sample (Wujeska-Klause et al., 2019). Anthocyanins were determined by recording the absorbance at 532 nm and 653 nm. Anthocyanin absorbance was normalised for the A_{532} due to chlorophyll by subtracting 24% of the absorbance at the chlorophyll maximum of A_{653} from the A_{532} (Murray and Hackett, 1991). The absorbance values due to anthocyanins were assumed to be directly proportional to the molar concentration of anthocyanins in the extracts. Therefore, absorbance indicated anthocyanin content in the samples (Murray and Hackett, 1991).

The wet weight measurements of each plant part type were converted into dry weight values using pre-determined wet-weight : dry-weight conversion ratios. All pigment measurements were standardised per gram dry weight. All pigment measurements were conducted within 5 hours to minimise pigment degradation.

Trapping structure characterisation

To assess trapping structures as a possible adaptive trait in *U. stellaris*, the physical structure of traps was examined using scanning electron microscopy. Before examination, freshly excised traps of varying ages were fixed in a mixture of 2.5% glutaraldehyde in a 0.1 M phosphate buffer. Trap material was washed in a 0.1 M sodium phosphate buffer and dehydrated using a graded ethanol series. Traps were then subjected to critical-point drying using CO_2 and sputter-coated with gold. Samples were examined using a TESCAN Vega Oxford TS 5136LM Electron microscope of the Electron Microscope Unit, Rhodes University, South Africa.

Changes in the proportion of functional traps per leaf with age were also determined. A leaf was excised from every fourth node along the stolon and shaken while submerged to induce

trap firing. Excess water was removed from this leaf, and the leaf was placed in a petri dish on a piece of moist filter paper. The petri dish was closed to keep the leaf from desiccating and placed in a growth chamber maintained at 25 °C in darkness for 5 hours to allow traps to reset. After 5 hours, the total number of traps was counted, the traps were manually triggered with the tip of a pin, and the total number of traps containing air bubbles was counted. The ability of a trap to fire and, therefore, trap functionality is represented by the aspiration of an air bubble into a trap (Adamec and Poppinga, 2016), the presence of which can be easily seen when viewing traps through a stereomicroscope. In addition, the number of traps per leaf was counted for one leaf at every fourth node along the stolon from the apical meristem. All microscopy was completed using an Olympus SZH-ILLD stereomicroscope.

Plant relative biomass investment

To assess whether plants invest significant resources into the production of traps, the relative biomass allocation to leaves, traps and stolons was determined. The biomass allocated to reproductive structures was not accounted for in this study, as reproductive structures do not arise from nodes predictably along the length of the stolon. A single leaf from every tenth node was excised, and the traps, leaf material, and subtending internode were dried separately on pre-weighed glass microscope coverslips at 80 °C to constant mass (Richards, 2001). The coverslips were then reweighed, and the mass of the plant tissues was calculated. All weight measurements were taken using a Mettler Toledo microbalance to four decimal places. To determine biomass investment in traps, the biomass of a subtending stolon, a single leaf from a node, and the traps on this leaf were determined. The leaf biomass and trap biomass measurements were multiplied by the average number of leaves per node to determine the total biomass allocation to structures per node.

Statistical analysis

Generalised linear models (GLMs) were used for all comparative analyses with various distributions and corresponding link functions dependent on the data distribution. Water chemistry and physical parameters were compared between sites using a GLM with a gamma distribution and inverse link function due to the non-normality of this continuous data.

For assessments of plant growth, successive nodes, as related to trap age, were treated as a categorical factor. To compare the internode length and leaf length between successive nodes, a GLM with a gamma distribution and inverse link function was used due to the non-

normality of the measurement data. To compare the number of leaves per node and the number of traps per leaf between successive nodes, a GLM with a Poisson distribution and log link function was used, as is applicable to count data. In these four analyses, the node number was nested within the site. “Functional traps” and “non-functional traps” were contrasted between nodes using a GLM with a binomial error distribution and a logit link function. All GLMs were run using the glm command from the package “stats” (R Core Team, 2023).

ANOVAs were run for each model using the Anova command from the package “car” (Fox and Weisberg, 2019). Estimated marginal means were obtained using the emmeans command from the package “emmeans” (Lenth, 2023). All statistical analyses were conducted in R (R Core Team, 2023). All means are reported in the form (mean±SE). The mean pH value reported was calculated as the proton activity mean.

Results

Water chemistry assessment

The measured water chemistry parameters indicate that sites are variable in terms of water chemistry (Table 2.1), with all water chemistry parameters differing significantly between sites, except for NH_4^+-N, PO_4-P and total phosphate. NO_3^--N is the dominant form of mineral N available.

Table 2.1: Mean and range of water chemistry parameters based on measurements taken at 15 *U. stellaris* sites. (*) denotes a significant difference of a parameter between sites.

Parameter	Unit	Mean	SE	Range
pH	-	6.71*	0.05	4.46-7.98
Conductivity	µS	336.29*	19.25	69.9-648
TDS	ppm	230.24*	14.03	46.2-682
$NO3^-$-N	µg l^{-1}	800.64*	65.08	223.02-1446.52
$NO2^-$-N	µg l^{-1}	83.31*	14.10	3.62-247.20
$NH4^+$-N	µg l^{-1}	0.24	0.03	0.05-0.63
PO4-P	µg l^{-1}	3.43	0.15	2.38-6.62
N_t	µg l^{-1}	206.44*	16.23	62.97-381.31
P_t	µg l^{-1}	1.12	0.05	0.78-2.16

24-hour environmental fluxes

All three measured parameters exhibit distinct diel fluctuations (Figure 2.1), which fluctuate predictably based on how long water bodies were exposed to sunlight. Water temperature and dissolved O_2 concentration increase with daylight hours, reaching maxima at 14:00 and 16:00, respectively (Table 2.2). Following these peaks, each parameter declines steadily through the night, with dissolved O_2 reaching a minimum at 06:00 and temperature reaching a minimum at 08:00 (Table 2.2).

In contrast to these trends, dissolved CO_2 concentration declines throughout the daytime, reaching a minimum at 18:00 and increasing steadily throughout the night, reaching a maximum concentration at 08:00 (Figure 2.1; Table 2.2).

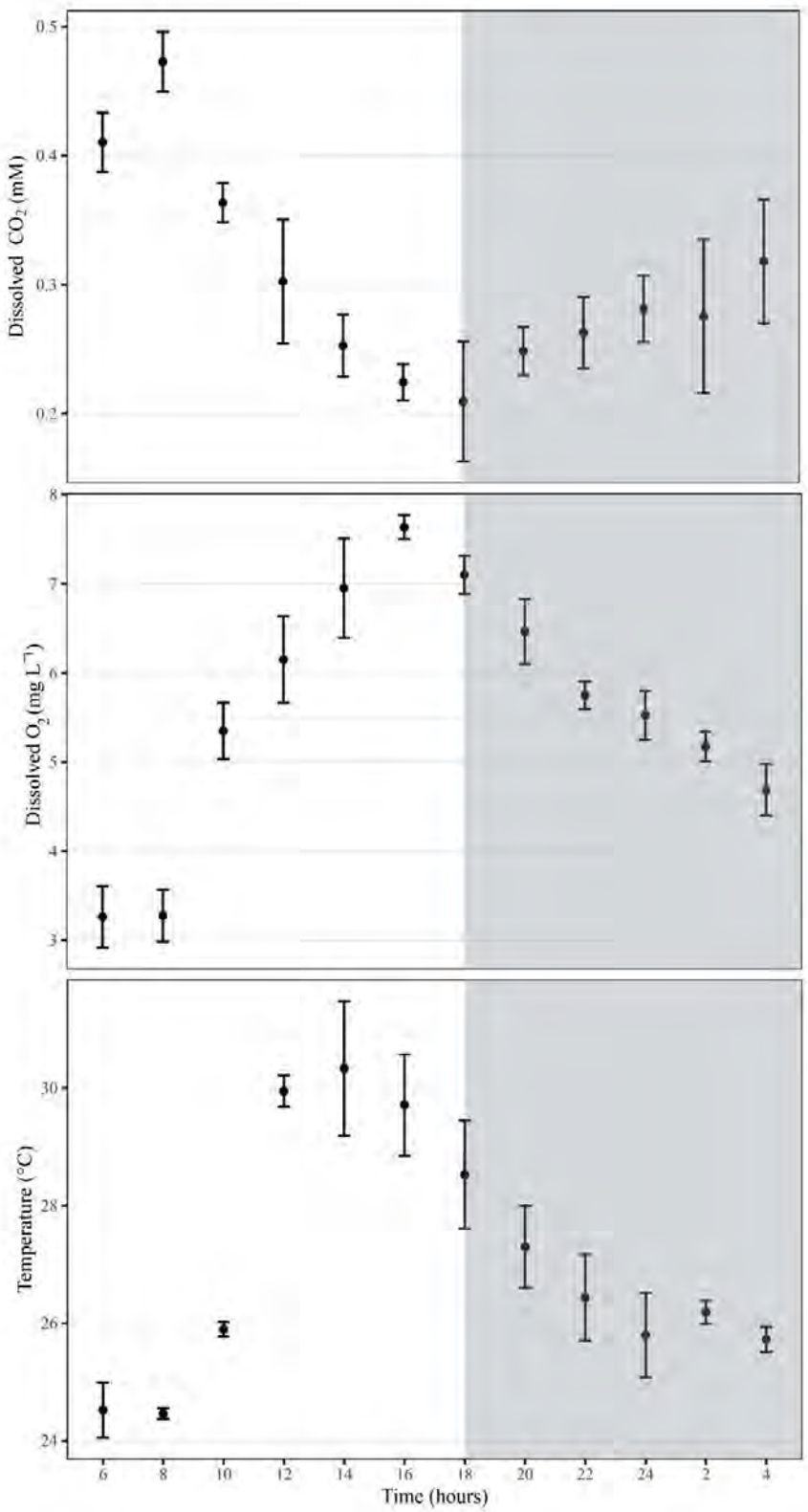

Figure 2.1: 24-hour water fluxes in dissolved CO_2, dissolved oxygen and temperature. Grey shading denotes the hours of darkness.

Table 2.2: Values of the minimum and maximum points reached by each measured parameter in situ.

Parameter	Unit	Minimum	Maximum
CO_2	mM	0.21±0.02	0.47±0.01
O_2	mg l^{-1}	3.26±0.15	7.64±0.06
Temperature	°C	24.46±0.04	30.34±0.48
pH	-	4.85±0.005	5.03±0.02

Plant growth form

Node number significantly influences leaf length (χ^2=38.586; df=5; P<0.0001). The length of leaves increases until node eight, after which leaf length reaches a maximum (Figure 2.2). From node eight to node 28, leaf length ranged from 47.05± 2.66 mm to 67.013± 4.68 mm. Additionally, node number significantly affects internode length (χ^2= 346.10; df=25; P<0.0001). Internodes increase in length from the apical meristem until node 10, after which there is no further increase in internode length (Figure 2.2). Maximum internode length ranges from 11.42± 0.79 mm to 17.83± 1.45 mm.

Node number does not significantly affect the number of leaves per node (χ^2=2.161; df=1; P= 0.14160). There is an average of 6.03±0.09 leaves per node throughout the length of the plant.

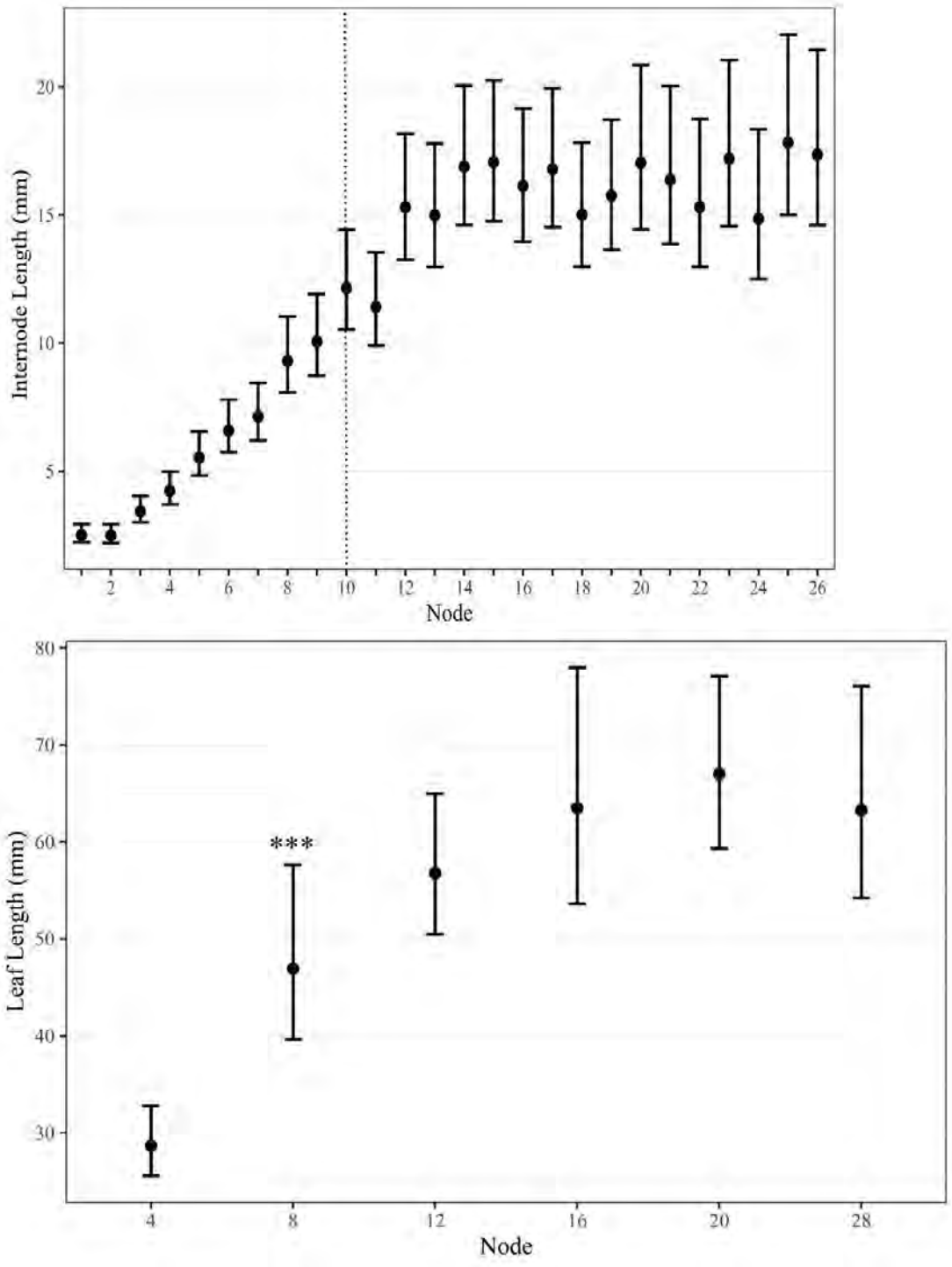

Figure 2.2: Internode and leaf length of *U. stellaris* with increasing node number. Significant differences in the length of leaves between successive nodes is indicated by: ***: P<0.001. The dotted line indicates the node after which there is no further significant increase in internode length.

Pigment assessment

The concentration of chlorophyll *a* per gram plant material is significantly higher in leaf material than in stolon and trap material in young and mature plant structures ($P<0.0001$) (Table 2.3). The concentration of chlorophyll *b* per gram plant material is also significantly higher in leaf material than in stolon and trap material in young and mature plant structures ($P<0.0001$) (Table 2.3). There are no significant differences in the chlorophyll *a* and chlorophyll *b* concentrations in the same structures of different ages except for chlorophyll *a* present in leaves, which showed a significant decrease in chlorophyll *a* concentration with age ($P=0.0389$) (Figure 2.3).

The concentrations of anthocyanins in young stolon material is significantly higher than that in leaf ($P=0.0011$) and trap material ($P=0.0017$) (Table 2.3). In mature material the anthocyanin concentrations in stolon ($P=0.0071$) and trap ($P=0.01$) material significantly exceed those in leaf material (Table 2.3), indicating an increase in anthocyanin pigments present in trap tissue with age (Figure 2.3).

Table 2.3: Pigment content of *U. stellaris* structures in young and mature plant material per unit dry weight. (*) denotes significant differences between different structures of the same age.

Structure	Chlorophyll *a*	Chlorophyll *b*	Anthocyanin Absorbance
	$\mu g\ g^{-1}$	$\mu g\ g^{-1}$	A_{532}-0.24[A_{653}]
Young:			
Leaf	163.71±0.67*	47.87±0.54*	1.20±0.17
Stolon	34.37±0.43	9.85±0.31	8.39±0.20*
Trap	27.40±0.44	9.98±0.39	1.41±0.37
Mature:			
Leaf	134.05±1.18*	41.57±0.54*	2.11±0.38*
Stolon	21.87±0.45	8.00±0.51	8.38±0.21
Trap	17.13±0.30	7.48±0.16	7.83±0.49

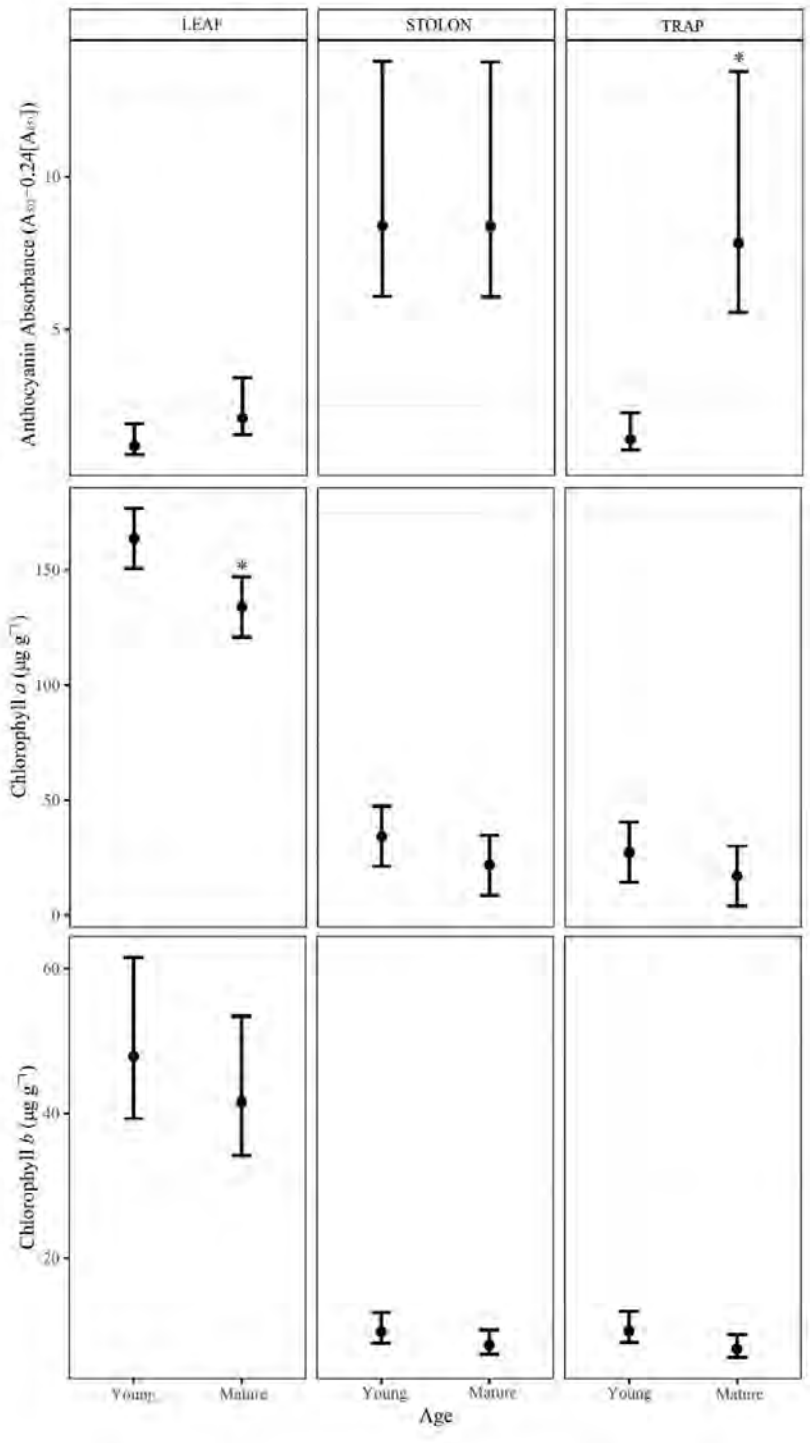

Figure 2.3: Pigment concentrations in *U. stellaris* leaf, stolon and trap material of two different ages, young material from node 13 and mature material from node 20; n = 8. (*) denote significant differences between the same structure of different ages. All measurements are standardised per unit dry weight. SE used for error bars are values taken from the GLM output, illustrating both variability in the data and uncertainty in the model parameters.

Trapping structure characterisation

U. stellaris produces hollow discoid bladder traps that connect to the main body of the plant with stalks visible at their bases (Figure 2.4). Traps differ in morphology as they mature. Young, non-functional traps are smaller than mature traps and are more rounded in appearance. The entrance to the trap is blocked by the ventral region of the trap, which is curved over the entrance (Fig. 2.4a). The external surface of the trap is covered in small, rounded glands (Kaur Cheema et al., 1992). Once mature, the dorsal region of the trap recurves upwards, exposing the entrance to the trap. In addition, a pair of finely branched, setiform appendages to either side of the entrance mature and extend upwards. Stiff setae, or bristles, in the central region outside the door extend outward from the entrance. Glands are still present on the external surface of the trap (Figure 2.4b). As traps age, the tissue making up the trapping structure begins to degrade. Breakages in the trap wall appear, and pieces of bristles and appendages start to be lost. Far fewer external glands are present on the external surface of ageing traps (Figure 2.4c).

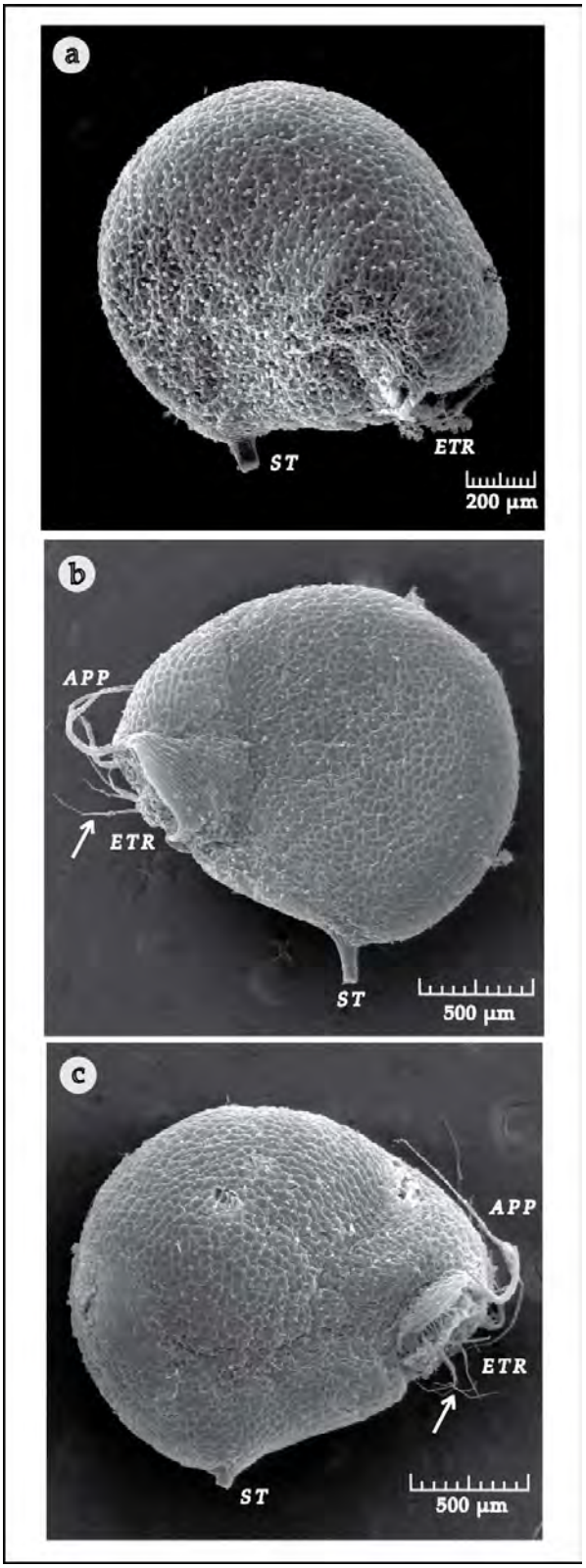

Figure 2.4: SEM images showing the structure of the traps of *U. stellaris* when young (a), mature (b) and old (c): Stalk (St). Entrance (Etr), Appendages (App) and Bristles (arrows).

Node number significantly affects the proportion of functional traps per leaf (χ^2=358.62; df=5; P<0.0001). Between nodes 4 and 12, there is an increase in the proportion of functional traps per leaf (P<0.05) (Figure 2.5). This is followed by a period of stability between nodes 12 and 20, where there are no significant differences between nodes (P>0.05), representing a peak in trap functionality. This peak is followed by a significant decline in the proportion of functional traps between nodes 20 and 28 (P=0.0106).

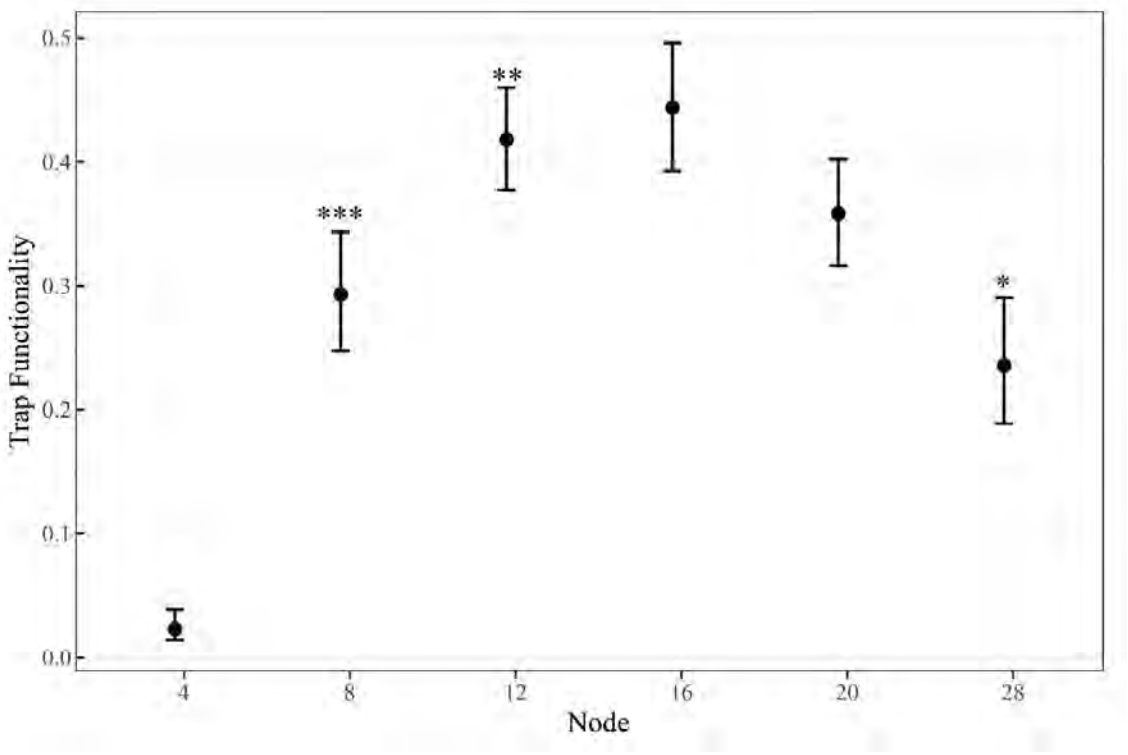

Figure 2.5: The proportion of functional traps per leaf at different nodes. Significant differences in the proportion of functional traps between successive nodes are indicated by: ***: P<0.001; **: P<0.01; *: P<0.05.

Node number (χ^2=12.599; df=5; P=0.02744) significantly influenced the number of traps per leaf. There is only a significant difference in the number of traps per leaf between nodes 16 and 20 (P=0.0005) (Figure 2.6), revealing that the leaves at nodes 20 and 28 have significantly fewer traps per leaf compared to the leaves of node 16 and all those further proximal to the apical meristem.

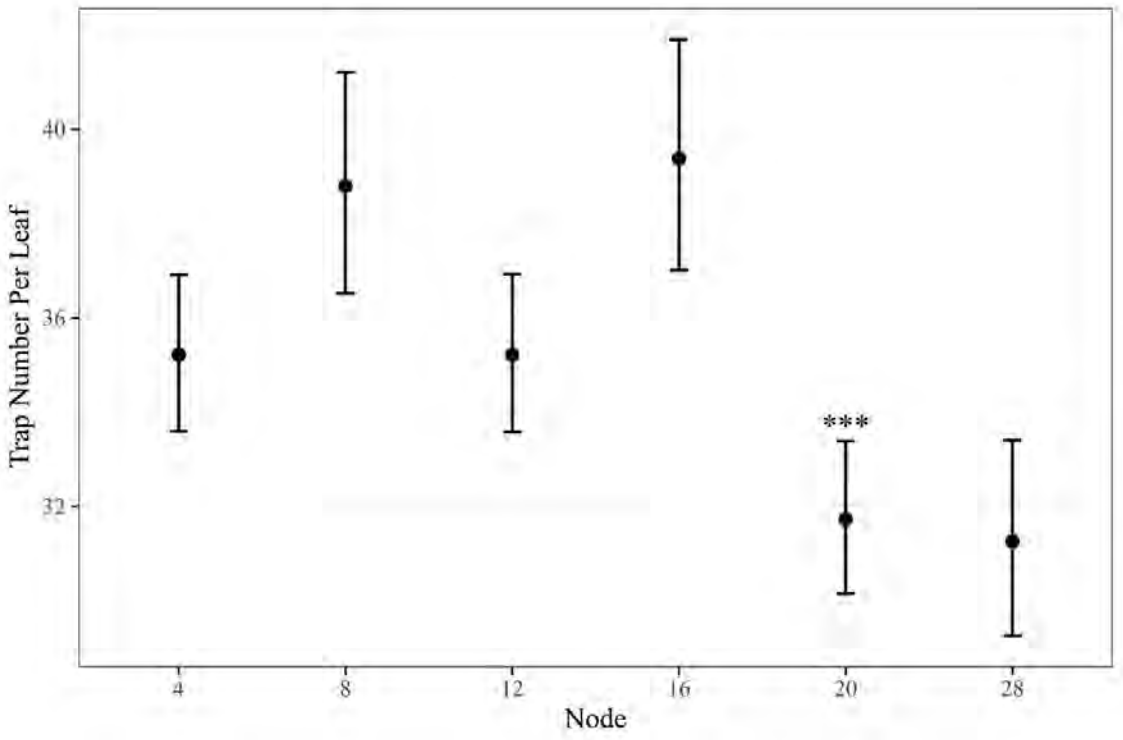

Figure 2.6: The number of traps per leaf at successive nodes along the length of the stolon. Significant differences in the number of traps per leaf between successive nodes are indicated by: ***: P<0.001.

Relative biomass allocation

The proportion of biomass allocated to trapping structures per node did not differ between nodes of a single plant (χ^2=4.8314; df=2; P=0.08931), with an average of 29.82% of total biomass being allocated to traps per node (Table 2.4).

Table 2.4: The average percentage of biomass allocated to trap, leaf and stolon material per node.

Structure	Mean	SE	Range
Trap	29.82	1.17	0-73.91
Leaf	62.61	1.18	21.47-96.54
Stolon	7.57	0.26	1.09-17.86

Discussion

Lentic freshwater systems are characterised by a specific suite of environmental conditions, several of which were documented in the water bodies where *U. stellaris* has been observed to grow. These water bodies fall comfortably within the standardised pond definition (Richardson et al., 2022): based on observation while in the field, these ponds are shallow, not exceeding 3 meters deep, small, not exceeding 1.5 ha in area, and have a low level of emergent vegetation coverage. Many sites investigated are permanent water bodies, but some, particularly small, shallow sites, are temporary. These ponds are closed systems relying on groundwater or rainfall runoff as water sources, with no through-flow, and are all relatively sheltered. These water bodies are largely stagnant, water stirring only resulting from ripples caused by wind or the presence of animals wading into pools. Despite being visually biotically active, water clarity is generally high, lending itself to relatively high light penetration. With all ponds being at most three meters in depth, the bottom sediments of pools are often clearly visible from above.

Diel fluctuations

The stagnant, biotically active nature of the habitats of *U. stellaris* drives distinct diel fluctuations in water conditions (Keeley, 1998). The shallow, clear water allows for high levels of photosynthetically active radiation to reach macrophytes and phytoplankton, which generally constitute a large proportion of the total biotic biomass (Keeley, 1983), causing fluctuations due to the consumption and release of gases involved in photosynthesis and respiration.

From first light, photosynthesis can proceed and photosynthetic organisms in the water column begin to assimilate dissolved CO_2. Due to the limited rate at which gases can diffuse through the water column from the layer of water exposed to the atmosphere (Marion, 2008), the dissolved CO_2 being drawn out of the water column by photosynthetic activity is not replaced at as great a rate at which it is being consumed. This diffusive limitation results in an initial decline in dissolved CO_2 concentrations, a decline that lessens as photosynthesis begins to be limited by the availability of CO_2 in the water column. Therefore, the dissolved CO_2 concentrations detectable in the water column decrease with increasing daylight hours. The maximum values of dissolved CO_2 measured throughout the day are similar to those documented elsewhere. However, the minimum does not fall to concentrations as low as those recorded by Pokorný and Květ (2007). The availability of CO_2 in the ambient water of

ponds of *U. stellaris* is high enough throughout the day to theoretically sustain a high rate of photosynthesis in these plants.

Mirroring this CO_2 decline are the dissolved oxygen concentrations in the water, which increase throughout the day as this gas is released as a byproduct of photosynthesis. The population of photosynthetic organisms in ponds is large enough that oxygen production is not masked by heterotrophic respiration's immediate consumption of this oxygen. Once again, the range of dissolved O_2 concentrations is similar to those recorded elsewhere (Pokorný and Květ, 2007). However, the range measured here is somewhat narrower, not reaching concentrations as high or as low as those documented in other ponds (Pokorný and Květ, 2007). These range limitations reflect productivity limitations in the system, which is likely not at the maximum potential level theoretically possible, which is an issue in freshwater systems often caused by nutrient limitation. The comparatively lower upper limit of oxygen concentrations recorded here may convey inadvertent benefits to macrophytes, with higher concentrations of dissolved O_2 favouring photorespiration. On the other end of the range, the threshold for anoxia in freshwater is a dissolved oxygen concentration of less than 0.1 $mg.L^{-1}$ (Pokorný and Květ, 2007). This threshold lies far below the minimum concentrations of oxygen measured in the pond assessed.

As the sun sets and photosynthetically active radiation is no longer available, photoautotrophs switch from photosynthesis to aerobic respiration. The respiratory capacity of photosynthetic organisms is combined with that of heterotrophs, and dissolved CO_2 concentrations in bulk water increase steadily (Keeley, 1998). The decline in dissolved oxygen concentrations throughout the night further illustrates this aerobic respiratory activity.

Concentrations of dissolved CO_2 in freshwater systems generally range from 0 to 0.5 mM (Madsen and Sand-Jensen, 1994; Van et al., 1976). As evidenced by the results reported here, CO_2 concentrations in the pond assessed are in the higher part of the reported range, even at a minimum just before sunset. High free CO_2 concentrations in ambient water are consistent with measurements taken in habitats of another species of aquatic *Utricularia* (Adamec, 2009). Based on these measurements, CO_2 availability in the bulk water seems sufficient to avoid substantial limitations to macrophyte photosynthesis, although rates achievable likely vary at different times of day in response to fluctuating levels of dissolved CO_2. The CO_2 concentration at which photosynthesis reaches a maximal rate in *U. stellaris* requires further investigation before the effects of these diel fluxes can be fully understood.

In addition to the diel fluctuations of dissolved gases, the temperature of the ambient water undergoes notable diel fluctuations. Measurements were taken in summer, reflected by the high temperatures recorded by early afternoon. These temperatures decline steadily throughout the afternoon in response to the decline in solar radiation and continue to fall throughout the night as heat is lost from the water to the cooler atmosphere.

Temperature is known to significantly increase the growth rates of aquatic macrophytes, influencing both photosynthetic rates and the availability of nutrients being released from sediments due to sediment respiration (Zhang et al., 2019). In several submerged aquatics, the optimal temperatures for maximum photosynthetic rates fall between 25 °C and 32 °C (Santamaría and van Vierssen, 1997), although exact optima are species-specific. During summer, temperatures of the ambient water of *U. stellaris* habitats during all daylight hours fall within this range, with even the minimum nighttime temperatures only falling below the lower threshold by half a degree. These temperatures support high rates of photosynthesis throughout the summer months and do not hinder the productivity and growth rates of *U. stellaris*. Based purely on observation in the field, it appears that *U. stellaris* experiences a decrease in growth rates during winter. This was evidenced by the substantial colonisation of periphyton covering plant tissues, even at the growing tip, during these months. In aquatic *Utricularia* species, these periphyton communities are usually kept to a minimum on young tissue during summer months due to rapid growth rates, tissue senescence, and regrowth (Friday, 1989). The decrease in growth rates is likely a direct effect of water temperatures limiting photosynthetic rates at this time of year, although this correlation demands verification.

Water nutrient status

Freshwater systems are placed into categories based on the nutrient status and correlated productivity of a system. The categories include oligotrophic, mesotrophic, and eutrophic, with water bodies being classified according to concentrations of the limiting nutrient, water chlorophyll content, and water transparency (Istvánovics, 2009). Based simply on the measurements of total nitrogen and total phosphorus documented in this study, the nutrient status of the habitats of *U. stellaris* is low, indicating that the sites fall comfortably into the category of oligotrophic (Smith et al., 1999; Vollenweider and Kerekes, 1982). The sites assessed contained less than 10 μg l^{-1} total phosphorus, and the mean total nitrogen was less than 350 μg l^{-1}, with only a single measurement from a single site being above this threshold

and falling into the range for mesotrophic systems. These results support the hypothesis that sites of *U. stellaris* are nutrient-poor, a recognised characteristic of the habitats of aquatic *Utricularia* (Guisande et al., 2007; Juniper et al., 1989).

Oligotrophic systems are characterised by a lack of mineral nutrients, which results in limitations to the growth rates of biota in the system. Water bodies classified as oligotrophic have high water clarity and a lack of productivity. This low productivity generally lends itself to constancy regarding water parameters, usually not characterised by distinct diel cycles (Pokorný and Björk, 2010). The site of *U. stellaris* assessed does not appear to be lacking in productivity, with distinct productivity-driven diel fluctuations being documented, indicating high productivity in this system despite nutrient limitation.

Water pH

The pH scale is another important water parameter and a widely utilised way of representing the acidity and basicity of a water body. The pH of a water body influences the biological processes that can occur in that water body, and the processes that take place, in turn, affect water pH (Boyd, 2000b). The most suitable range of pH for the success of aquatic life lies between pH 6.5 and pH 9, spanning from slightly acidic to very basic conditions (Boyd, 2000b). The pH measures taken in ponds of *U. stellaris* fall partly out of this range, some being more acidic than is optimal. One of the most important factors influencing the pH of water bodies is the concentration of dissolved CO_2. On dissolution, CO_2 reacts with water to form carbonic acid (Stumm, 1995), an acid that can draw pH down to approximately 4.5. A pH below this level indicates the presence of another, stronger acid (Boyd, 2000b). The lower extreme of the pH range measured here is 4.46, just lower than the 4.5 carbonic acid cutoff. Therefore, the sites at which low pH was measured may be simply very high in dissolved CO_2, as in the pond in which diel CO_2 fluxes were quantified. At any pH below 5, CO_2 is the only type of inorganic carbon meaningfully available to photoautotrophs. Above a pH of 5, the proportion and availability of the bicarbonate inorganic carbon source increase with increasing pH until approximately pH 8.3, at which CO_2 becomes unavailable, and the only inorganic carbon source remains as bicarbonate (Boyd, 2000b). None of the pH readings taken reaches the alkalinity of pH 8.3, indicating that CO_2 is always available in the ambient water to some extent, even if concentrations are low.

The pH values measured here suggest high levels of dissolved CO_2 available within ponds where *U. stellaris* grows, with this remaining the primary source of inorganic carbon. These

findings, in conjunction with the diel CO_2 fluctuation measurement, support the hypothesis that the CO_2 concentrations in the ambient water of the habitats of *U. stellaris* are high, as in the habitats of other aquatic *Utricularia* species (Adamec, 2008; Moeller, 1978).

Water mineralisation

Water conductivity and total dissolved solids (TDS) are both measures of a water body's mineralisation level. Most commonly, dissolved minerals in freshwater systems consist of calcium and magnesium, the determinants of water hardness, and sodium, potassium, bicarbonate, and others (Boyd, 2000a). Generally, a freshwater system is defined as having a TDS of less than 1000 ppm and a conductivity of less than 700 µS/cm (Rhoades et al., 1992). The maximum value of TDS measured in ponds of *U. stellaris* was 628 ppm, while the maximum conductivity value was 648 µS/cm. Therefore, the ponds of *U. stellaris* assessed can be defined as freshwater systems, as opposed to brackish or saline conditions with a higher level of mineralisation. With an increase in the TDS of the ambient water comes an increase in the osmotic pressure experienced by aquatic organisms (Boyd, 2000a). At a certain point, this pressure would result in water loss from the cells of organisms as water diffuses from an area of high water concentration to an area of lower water concentration, the ambient water. Due to the reasonably low mineralisation levels in the ponds assessed, osmotic pressure is not detrimental to organisms occupying these habitats.

Environmental conditions that warrant adaption

Dissolved CO_2 concentrations in the bulk water of habitats of *U. stellaris* are consistently high. Even though ambient CO_2 concentrations do not necessarily indicate the CO_2 concentrations available in the boundary layer of water surrounding leaf surfaces, the current dataset indicates that limitations are unlikely. Another factor governing photosynthetic rates is ambient water temperatures. Summer temperatures are not limiting, falling within the optimal range documented in aquatic macrophytes throughout the day. However, winter temperatures appear to negatively impact plant productivity and growth rates, potentially resulting in plant dormancy during winter months. Productivity and growth rates are also influenced by the low nutrient availability in the ambient water, with the sites of *U. stellaris* being classified as oligotrophic. The low nutrient concentrations in *U. stellaris* habitats support the hypothesis of this study, and these findings are consistent with what is known about the habitats of aquatic *Utricularia* (Guisande et al., 2007; Juniper et al., 1989). Low nutrient availability limits productivity and is likely a driver of adaptation in this habitat.

Plant growth form and potential adaptations

The first evident form of adaptation to the aquatic habitat is morphological, with *U. stellaris* bearing many highly dissected leaves, with an average of 6 leaves produced per node, which branch further into secondary and tertiary leaf segments. These leaves are extremely fine and delicate, only a few cell layers thick, minimising self-shading and maximising the surface area exposed to light and dissolved gases in the ambient water (Reut et al., 2021), emphasising their primarily photosynthetic function. The photosynthetic function is further confirmed by the high chlorophyll *a* and *b* concentrations in leaf tissue compared to other plant structures. The substantial investment into photosynthetic output in these plants is clearly illustrated by the 63% of total biomass allocated to leaves.

Another notable morphological feature in *U. stellaris* is the absence of rooting structures, with a free-floating growth form. As mentioned, this growth form allows plants to maximise exposure to light and dissolved gases (Reut et al., 2021), which are present in greater abundance near the water's surface. In addition, this growth form allows for these plants to remain in the favourable upper layer of water even after a potential influx of water to the system. This growth form is well-suited to the closed, lentic systems in which *U. stellaris* has been documented to grow as current and through-flow would result in these plants being displaced. Therefore, *U. stellaris* exhibits adaptive traits that allow it to succeed in the aquatic environment. The lentic nature of ponds is potentially a critical governing factor determining whether *U. stellaris* is present in a water body.

Generally, non-rooted macrophytes are found in conditions where water nutrient levels are high (Lacoul and Freedman, 2006), with these plants only being able to take up nutrients in the ambient water. Therefore, the presence of unrooted *U. stellaris* in the low-nutrient sites examined here seems counter-intuitive. However, aquatic *Utricularia*, in addition to their high nutrient reutilisation efficiency (Adamec, 2008), are able to capture and breakdown zooplankton prey, which results in the supplementation of nitrogen and phosphorus (Adamec, 2018; Ellison and Gotelli, 2009). However, studies have shown remarkably low zooplankton trapping rates in comparison to the proportion of traps that contain living communities of microorganisms, including phytoplankton, bacteria, ciliates, fungi, and rotifers (Richards, 2001; Sirová et al., 2009). The ubiquity of these communities and the observed success of trap communities has led to the hypothesis that the benefits derived from living microbial trap communities may be of equal, if not greater, nutritional importance to *Utricularia*.

Utricularia stellaris, like other members of the genus, produce these trapping utricles, which are borne at the bases of secondary and tertiary leaf segments. In other species of aquatic *Utricularia*, biomass allocation to these utricles generally ranges from 10-55% of total vegetative biomass (Porembski et al., 2006; Richards, 2001). The biomass allocation to traps in *U. stellaris* falls within this range, with an average of 30% of total biomass per node allocated to trapping structures. This high investment suggests that the production of traps must convey some benefit to plants that outweighs the substantial costs of production.

As in leaves, developing and maturing with age, trapping structures experience a similar form of growth from the onset of development at the growing tip of the plant, not changing in the number of traps per leaf, but changing in size and, importantly, functionality. Traps are initially small, transparent, green, and nonfunctional, with the trap door inaccessible and still lacking the ability to create negative pressure within traps. On reaching maturity, the trap door becomes accessible. The negative pressure that can now build up in traps allows for the suction and firing function of traps to become active and traps begin to capture suspended organisms and detritus in the surrounding water. Traps of *U. stellaris* do not remain functional for long. With an accumulation of anthocyanin pigments in the tissues of traps, senescence sets in, and trapping structures lose functionality and are lost from leaves not long after. This short period of trap functionality is similar to that found in studies of other aquatic *Utricularia* species (Friday, 1989). The loss of utricle structural integrity is evident in the degradation of trap wall integrity, the fraying and loss of appendages and bristles, and a decline in the number of external glands shown in *U. stellaris*. All of these factors contribute to a decrease in the ability of the trap to create an isolated internal environment and create and maintain negative pressure effectively enough to allow for trap firing. The complex trapping action of the utricles of *U. stellaris* suggests that they are important for capturing what is present in the water column. However, the beneficial nature of that which is captured remains unconfirmed.

With the maturation of the utricle structure comes an in-tandem maturation of appendages and bristles surrounding the trap door region. As utricles mature, these finely branched structures extend outwards, creating a feathery network surrounding the trap door. These structures increase zooplankton capture, leading free-swimming organisms out of the open water and inwards towards the trap door (Horner et al., 2018; Meyers and Strickler, 1979) and are also a source of internal trap microbes following the colonisation of appendages by periphyton (Richards, 2001; Sirová et al., 2009). Both potential appendage functions lend

themselves to capturing organisms, be it zooplankton prey or microbes. These results suggest that capturing living organisms is an essential function of trapping structures. However, the definite benefits derived from these subsets of organisms remain unclear.

Based on the assessment of *U. stellaris* pond environmental conditions, the main limitation to macrophyte success appears to be the limited availability of nutrients in these systems. However, this supposed limitation has little influence on the productivity of these plants, as is evidenced by observed rapid growth and the high productivity of these typically low-productivity oligotrophic systems. In addition, they appear to lack the apparent strategies that would allow plants to overcome these limitations to such an extent that these productivity levels could be supported. Therefore, the benefits derived from trapping structures may alleviate nutrient limitation.

Conclusion

The primary environmental constraint opposing productivity in *U. stellaris* is low nutrient availability. In theory, these nutrient limitations should limit plant productivity. However, *U. stellaris* does not exhibit the expected adverse effects of these limitations. *U. stellaris* may be able to overcome these limitations by producing traps, which constitute a high proportion of total biomass and must, therefore, provide some benefit to plants that outweigh the costs of their production. The structure and mechanism of action of these traps suggest that they are beneficial for what they capture from the surrounding water column, contents potentially supplementing nutrient uptake in these plants. The relative contributions of zooplankton and microbial communities to this nutrition, and whether microbial communities convey any nutritional benefit to plants remains unresolved.

Chapter 3: The negligible contribution of trap tissue to alleviation of CO_2 limitation in the submerged aquatic plant *Utricularia stellaris* L. fil. (Lentibulariaceae)

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Introduction

The autotrophic transformation of inorganic carbon and water into organic carbon compounds rich in energy is the basis of many trophic interactions and cannot occur without inorganic carbon uptake. While terrestrial macrophytes can take up this inorganic carbon in the form of carbon dioxide (CO_2) directly from the atmosphere, inorganic carbon is not as readily accessible to submerged macrophytes. These plants access atmospheric CO_2 only once it has bridged the air-water interface. The rate at which this transfer occurs is mainly dependent on the turbulence of surface waters (MacIntyre, 1995), turbulence being modulated by factors such as the size of a water body, limiting the impact of wind on surface water motion (Woolf, 2005). Regardless of the extent of surface water turbulence, some level of dissolution of atmospheric CO_2 does occur, forming carbonic acid, which further dissociates into various ions, including bicarbonate (HCO_3^-) (Stumm, 1995). Reactions and dissociations occur in water at a rate that attempts to reach an equilibrium between these reactants in solution and an equilibrium between dissolved CO_2 and atmospheric CO_2.

Despite the tendency of gases to be exchanged towards an equilibrium, the dissolved CO_2 concentrations in freshwater bodies are rarely equivalent to those in the atmosphere. This is due to the slow rate of CO_2 flux across the air-water boundary and the action of biota occupying the water body. Organisms take up CO_2 or HCO_3^- during photosynthesis and releases CO_2 as a byproduct of respiration and oxidation of organic matter (Stumm, 1995). Because photosynthesis only takes place when photosynthetically active radiation is available, some water bodies, particularly small, shallow, stagnant pools, are often driven away from gaseous equilibrium with the atmosphere and are instead characterised by significant diel fluctuations in CO_2 availability (Keeley, 1998).

In small lentic systems where water turbulence is minimal, the movement of dissolved inorganic carbon through water largely relies on diffusion alone, moving from the carbon source to the site of CO_2 uptake. The movement of molecules through a medium as viscous as water is not a rapid process, with CO_2 having been reported to diffuse 10^4 times more slowly in water than in air (Marion, 2008). Even in well-stirred systems, an unstirred boundary layer of water remains around the leaf surface which causes low CO_2 availability at the leaf's surface, forming a significant resistant force opposing CO_2 fixation in aquatic macrophytes (Smith and Walker, 1980). The adverse effects of this boundary layer on CO_2 fixation have been shown not only in lotic systems but also in conditions where CO_2 availability in ambient water is high (Black et al., 1981). Even though the decline in the free CO_2 concentrations of bulk water following sunrise is gradual, CO_2 available to plants in the leaf boundary layer is likely depleted rapidly from the time photosynthetically active radiation becomes available (Smith and Walker, 1980), regardless of high dissolved CO_2 concentrations in the ambient water.

In response to CO_2-limited conditions, submerged freshwater macrophytes have evolved strategies to overcome diffusive CO_2 limitations and their adverse effects on photosynthetic rates. One such group of macrophytes is plants in the genus *Isoetes,* which have evolved a diel acidity cycle similar to CAM photosynthesis. Plants can take up and store inorganic carbon at night when respiratory CO_2 is readily available. This carbon, stored in vacuoles as organic acids (Keeley, 1981), is then used for photosynthesis during the daytime when dissolved CO_2 concentrations are generally limiting (Suissa and Green, 2021). Several other lineages of aquatic macrophytes can take up carbon through their roots, where CO_2 concentrations often exceed concentrations around the leaves due to microbial mineralisation here (Maberly and Gontero, 2017; Søndergaard and Sand-Jensen, 1979). Other freshwater macrophytes can induce a facultative C_4-like metabolism with carbon-concentrating capabilities (Holaday and Bowes, 1980). Some plants are able to use inorganic carbon sources other than CO_2. Under alkaline conditions when CO_2 is scarce, HCO_3^- assimilation may be induced, this strategy of inorganic carbon gain being the most commonly employed physiological strategy in freshwater macrophytes (Maberly and Gontero, 2017). While, in certain habitats, plants have been documented to take up free amino acids as a carbon source (Krab et al., 2008), this phenomenon is not well explored or documented as a means to supplement carbon uptake in freshwater macrophytes.

Another type of adaptation to CO_2 limitation is morphological. Plants produce thin leaves, often only two cell layers thick, which is possible in the aquatic habitat where plant tissues are supported by the surrounding water. This trait allows the plants to maximise the surface area to volume ratio of plant tissue, increasing the potential of these leaves to take up dissolved CO_2 while allowing for a greater quantity of less costly surface area to be produced (Sculthorpe, 1967). These plants can avoid CO_2 limitation for a longer period than plants with thicker leaves as they require less CO_2 per unit surface area but can produce a net greater amount of photosynthetic surface area exposed to dissolved CO_2 (Maberly and Madsen, 2002).

Many physiological and biochemical mechanisms utilised to gain inorganic carbon under CO_2 limiting conditions are inducible and will be up-regulated when CO_2 is limiting (Maberly and Gontero, 2017). The prevalence of these adaptations indicates how commonly it is limiting to photosynthetic output in these systems.

A group of macrophytes that occur in such freshwater systems are the aquatic subset of the genus *Utricularia,* including the study species *U. stellaris*. Aquatic *Utricularia* species undergo rapid growth (Friday, 1989) which results in substantial biomass turnover and carbon loss (Adamec, 2008). In addition, these plants produce costly trapping structures that contribute little to photosynthate production (Adamec, 2006) and a portion of total photosynthates produced by the plant are released into the inner environment of these traps (Sirová et al., 2010). High photosynthetic rates in these plants are likely necessary to support rapid growth and trap production and maintenance (Adamec, 2018) and generally exceed those of non-carnivorous plants (Adamec, 2018, 2013, 2006). To achieve these high rates, ample CO_2 in ambient water is an essential ecological requirement for these plants (Adamec, 1999). A well-documented characteristic of the habitats in which aquatic *Utricularia* have been found to grow is high concentrations of dissolved CO_2 (>0.1 mM) in the ambient water (Adamec, 2018, 2009). This environmental characteristic was documented in one site of *Utricularia stellaris* that was assessed (Chapter 2). Therefore, based on the high concentrations of dissolved CO_2 available in the bulk water, the requirement for high availability of inorganic carbon sources seems to be met. However, these measures do not necessarily indicate the CO_2 available to plants in the boundary layer surrounding leaves, concentrations often lowered by diffusive resistances and diel CO_2 fluctuations.

Of the known strategies used by aquatic macrophytes ensure inorganic carbon availability only a few of these potentially aid aquatic *Utricularia* species. Most aquatic *Utricularia* are rootless and free-floating (Taylor, 1989). This means that they do not have the potential to obtain carbon from sediments through rooting structures. In addition, most aquatic *Utricularia* species investigated use dissolved CO_2 exclusively (Adamec, 1995; Adamec and Kovářová, 2006; Moeller, 1978) while a single species was found to utilise HCO_3^- only under extreme environmental conditions (Adamec, 2009) and is, thus, ecologically unimportant (Adamec, 2018). The most evident strategy to maximise CO_2 gain is simply through the growth form of aquatic *Utricularia*, with the plant producing a branching network of extremely fine, feathery leaves (Reut et al., 2021), maximising the plants "reach" into surrounding CO_2-containing water.

In *U. stellaris*, 30% of the total plant biomass consists of trapping structures (Chapter 2). These structures constitute more fine extensions of plant surface area into the surrounding water and, should they be photosynthetic, this biomass would undoubtedly increase the photosynthetic surface area exposed to dissolved CO_2. This raises the question of whether trapping structures contribute to the overall photosynthetic output of *U. stellaris.* If so, they may aid in maximising photosynthetic surface area and alleviate potential CO_2 limitations. These questions are particularly important in the aquatic species *Utricularia stellaris*, about which little is known regarding its ecophysiology.

This study aims to determine whether trapping structures contribute significantly to photosynthetic output. Based on the low chlorophyll concentrations of trapping structures (Chapter 2) and the low photosynthetic rates measured in the traps of other aquatic *Utricularia* species (Adamec, 2014, 2006), this study hypothesises that trapping tissue will not contribute substantially to photosynthetic output and that leaf material will be the primary site of photosynthesis.

In addition, this study aims to determine whether photosynthetic rates of *U. stellaris* are CO_2 limited in natural pond conditions. Based on what is known about concentrations of dissolved gases in aquatic systems, *U. stellaris* may be CO_2 limited in natural pond conditions due to both diel CO_2 fluctuations and diffusive resistances. If CO_2 concentrations are limiting, this limitation may result in a decline in the overall photosynthetic rate from the maximum theoretical rate achievable.

Methods and Materials

Photosynthetic rates of leaves, traps and stolons

The photosynthetic capacity of young and mature leaf, trap and stolon material of *U. stellaris* were assessed. Whole plants were collected from site 7 (Appendix A) on June 25th, 2023, and transported back to the laboratory for analysis. Five whole leaves were excised from nodes 13 and 20, with the former representing young plant material and those from node 20 representing mature plant material. To prevent the presence of air bubbles within traps, traps were stimulated to fire while submerged before excision. From these leaves, approximately 100 large traps were removed for assessment. All remaining trap material was excised and discarded. In addition, six lengths of stolon material were collected for both young and mature plant segments, the young stolon material being collected from node 13 working towards the apical meristem to node seven. The mature stolon material was collected from node 20, moving backwards down the stolon towards the senescing plant material to node 26.

For each set of plant parts, photosynthetic rates were measured in a Clark-type oxygen electrode chamber filled with 5 ml of distilled water bubbled with ambient air. The resultant dissolved CO_2 concentration of the distilled water was approximately 0.02 mM CO_2. The chamber was maintained at a temperature of 25 °C and a spin rate of 1556±46.97 rpm controlled by a stirring bar. Grow lights were placed in fixed positions around the chamber in a dimly lit laboratory to ensure light availability remained as constant as possible. The chamber remained at a photon flux density (PPFD) of 2000 μmol m^{-2} s^{-1}. Following calibration of the oxygen electrode, photosynthetic rates were measured for ten-minute intervals per structure. Measurements of each structure were made for material taken from eight different plants from the same site. Following measurements, the wet weight of all structures was recorded to allow for wet weight to dry weight conversion using predetermined WW : DW ratios. Measurements were represented per unit of dry weight and per unit of chlorophyll (Chapter 2).

Modelling in situ photosynthetic rates

CO_2 response curves were constructed for *U. stellaris* without separation of material into different structures. Three whole leaves, still bearing traps, and one length of stolon material were collected from node 13. All measurements were made in a Clark-type oxygen electrode maintained in the same conditions described above. Photosynthetic measurements of this material were made in a solution of 1 mM $NaHCO_3$ and 0.1 mM KCl (Adamec, 2013), which

was bubbled with CO_2 to achieve progressively higher concentrations of dissolved CO_2. Specific dissolved CO_2 concentrations were intended to be obtained while bubbling with CO_2 by tracking changes in pH. The intended concentrations were 0.02, 0.05, 0.10, 0.25, 0.5 and 1 mM dissolved CO_2. However, due to the difficulties associated with obtaining a solution of an exact pH, all prepared media were titrated against a 0.0227 N sodium hydroxide solution using a phenolphthalein indicator until an endpoint was reached. These titrations provide an exact dissolved CO_2 concentration to correlate with a specific measured photosynthetic rate. All photosynthetic rate measurements were taken for 10 minutes, after which the material was rinsed in distilled water and used for the following measurement in a solution of higher dissolved CO_2 concentration. Following measures, the wet weight of all material was recorded to allow for wet weight to dry weight conversion. Eight replicates of the CO_2 response curves were constructed with plant material from eight separate plants being used to form an individual curve.

To account for CO_2 diffusive resistances under stagnant water conditions, three whole leaves still bearing traps were excised from node 12. Photosynthetic rates of these leaves were measured in a Clark-type oxygen electrode under the same conditions as in previous photosynthetic measurements. Measurements were taken while leaves were submerged in a 1 mM $NaHCO_3$ and 0.1 mM KCl solution at both low (±0.1 mM) and high (±0.5 mM) dissolved CO_2 concentrations. No significant differences were detected between measurements taken at low and high CO_2 concentrations, so the collected data was pooled. Measurements were taken under three different levels of water motion, with spin rates of the stirring bar being changed to progressively slower speeds, from 1556±46.97 rpm to 789±68.38 rpm to 479±40.71 rpm.

The spin rate of the stirring bar was measured by connecting a reed switch to an LED using an Arduino circuit board. The reed switch was placed in contact with the coil, which produces the electromagnetic current that spins the stirring bar. The reed switch was activated every time the stirring bar made a rotation, and this activation resulted in the LED producing a flash of light. The number of times the light flashed per minute was counted for eighteen replicates at each spin rate. These measures were averaged to obtain a spin rate of the stirring bar.

At each spin rate, measurements of photosynthetic rates of plant material were taken for 10 minutes, after which the wet weight of plant material was recorded. The same plant material

was used for one replicate consisting of measurements at each spin rate. These replicates amounted to eight measurements of photosynthetic rates at each spin rate.

All photosynthetic rate measurements were represented per unit of dry weight and unit of chlorophyll (Chapter 2).

Statistical analysis

Photosynthetic rates were modelled with mixed effects models using the lmer command from the package "lme4" (Bates et al., 2015). ANOVAs were run for each model using the Anova command from the package "car" (Fox and Weisberg, 2019) and estimated marginal means were obtained using the emmeans command from the package "emmeans" (Lenth, 2023).

Individual CO_2 response curve replicates were each fitted by piecewise regressions using the lm command from the package "segmented" (Muggeo, 2008). From these models for each replicate, photosynthetic rates were predicted for CO_2 concentrations of 0.06, 0.1, 0.25, 0.5, 1 and 1.5 mM using the predict command from the package "stats" (R Core Team, 2023). The replicates at each CO_2 concentration were used to find an average value and 95% confidence intervals for each CO_2 concentration, and these averaged replicates formed a single response curve. To model the photosynthetic rates of plants during the hours of daylight *in situ*, each CO_2 response curve model was used to predict photosynthetic rates for various CO_2 concentrations using the predict command, as above. However, CO_2 concentrations that were input were those measured every two hours from 06h00 until 18h00 in situ (Chapter 2).

When plotting all replicates of photosynthetic rates according to the stirring speed of the stirring rod, the resultant curve followed an exponential distribution. This data was log-transformed to allow it to be modelled using a linear model using the lm command from the package "stats" (R Core Team, 2023). The resultant exponential coefficients were extracted from this model. Using these coefficients, substituted into an exponential equation, it was predicted what each raw value of the photosynthetic rate used to create the CO_2 response curves would theoretically be if the water stirring rate were set to zero when these measurements were taken. Each replicate of these downscaled values was then, once again, fitted by piecewise regressions to produce a photosynthetic CO_2 response curve representative of what rates would be if water were completely stagnant. Downscaled photosynthetic rates were then modelled at bihourly CO_2 concentrations measured in situ.

All statistical analyses were conducted in R (R Core Team, 2023). All means are reported in the form (mean±SE).

Results

Photosynthetic rates of leaves, traps and stolons

When measurements of photosynthetic rates were standardised per unit dry weight, it was found that the photosynthetic rates of leaves, traps and stolons did not differ between the same structures of different ages (χ^2=0.0190; df=1; P=0.8905). However, the photosynthetic rates of different structures of the same age did differ significantly (χ^2=50.4283; df=2; P<0.0001), with the photosynthetic rates of leaves exceeding both those of stolon and trapping material (Table 3.1, Figure 3.1). In young plant material, the photosynthetic rates of trap material reached only 27% of that in leaf material, and photosynthetic rates of stolon material reached only 0.2% of leaf material.

When standardised per unit chlorophyll *a*, there were no significant differences in photosynthetic rates between ages (χ^2=0.0037; df=1; P=0.95158) or between structures (χ^2= 6.5665; df=2; P=0.84379) (Table 3.1).

Table 3.1: Average net photosynthetic rate per unit dry weight (P_{NDW}) and net photosynthetic rate per unit chlorophyll (P_{Nchl}) of young and mature trap, leaf and stolon material of *U. stellaris* (mean ± SE). (*) denotes significant differences between different structures of the same age.

Structure		P_{NDW}	P_{Nchl}
		mmol h^{-1} kg^{-1}DW	mmol g^{-1}chl.h^{-1}
Young:			
	Leaf	1853.01±474.18*	1555.01±547.29
	Stolon	3.44±2.87	9.54±7.82
	Trap	499.98±222.23	3078.44±1609.19
Mature:			
	Leaf	1992.70±447.34	2028.72±535.05
	Stolon	9.82±9.82	105.76±105.76
	Trap	257.36±207.80	2351.88±2046.23

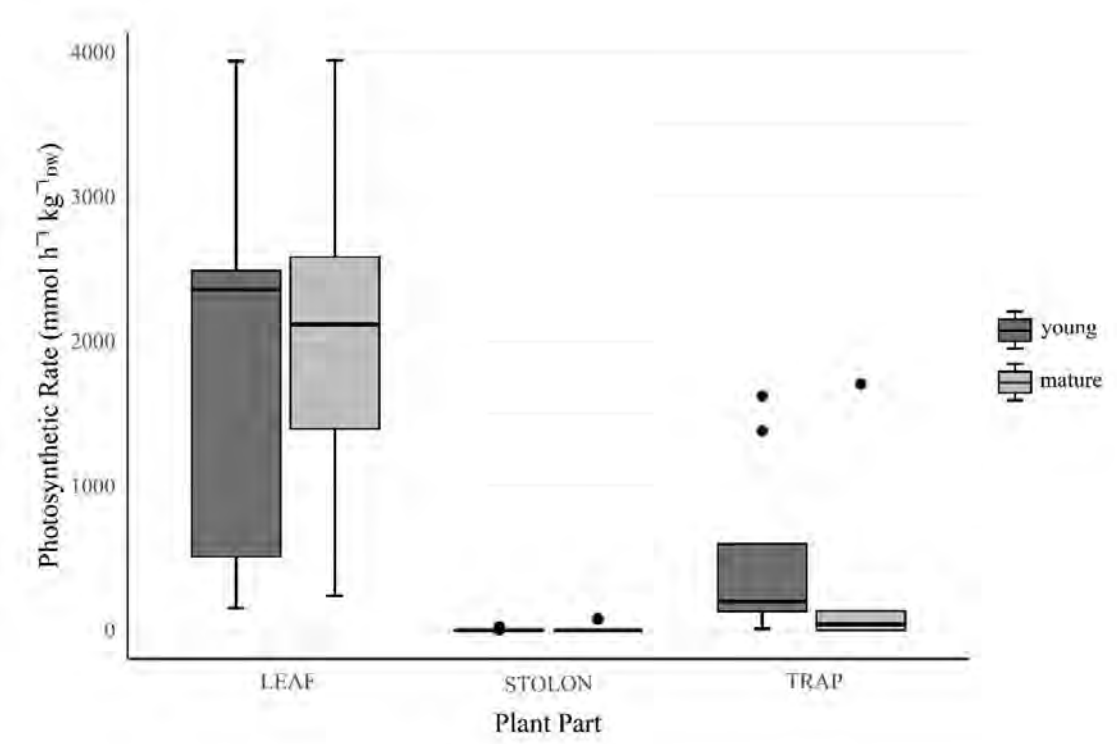

Figure 3.1: Photosynthetic rates of young and mature leaf, stolon and trap material of *U. stellaris*, standardised per unit dry weight. Points represent outliers, and lines transecting bars represent median values.

Modelling of photosynthetic rates in situ

The spin rates of the stirring rod in the oxygen electrode chamber were determined to be 479.17±40.71 rpm, 789.44±68.38 rpm and 1556.94±46.97 rpm with increasing speed. When modelling the rpm against the log-transformed photosynthetic rates, a linear relationship was obtained (Figure 3.2), and the relevant coefficients required for the exponential equation were extracted. These values were inputted into an exponential equation to downscale each measurement of photosynthetic rates used to produce the CO_2 response curves to what they would theoretically be if water were unstirred. These values were used to produce a CO_2 response curve at rpm=0 (Figure 3.3).

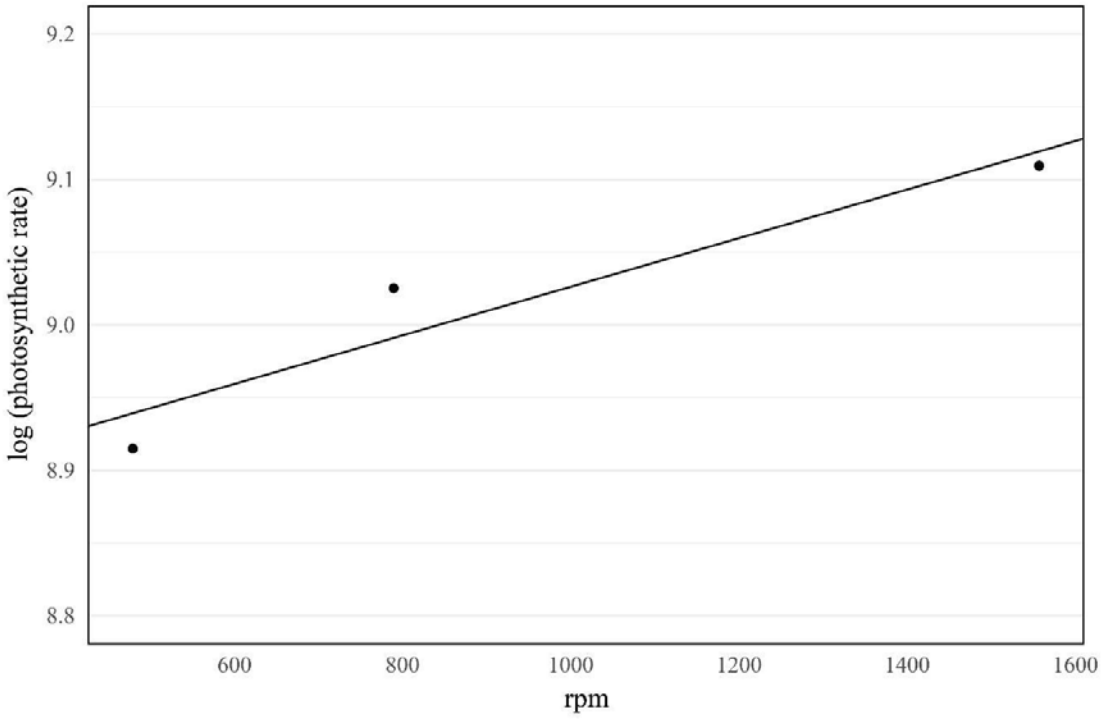

Figure 3.2: A plot of the linear model produced by log transforming the photosynthetic rates of *U. stellaris* measured at various spin rates of the stirring bar (rpm). Points represent the mean photosynthetic rate per rpm.

The resultant dissolved CO_2 response curves at rpm=0 and rpm=1556 do not correspond to the Michaelis-Menten model with a distinct biphasic turning point at 0.59±0.09 mM CO_2. This turning point lies beyond the maximum concentrations of CO_2 that were measured in natural pond ambient water (Chapter 2), as is represented by the dotted line (Figure 3.3).

The values of the curve constructed based on measurements taken under well-stirred conditions (1556 rpm) are higher than those modelled under stagnant conditions (rpm=0).

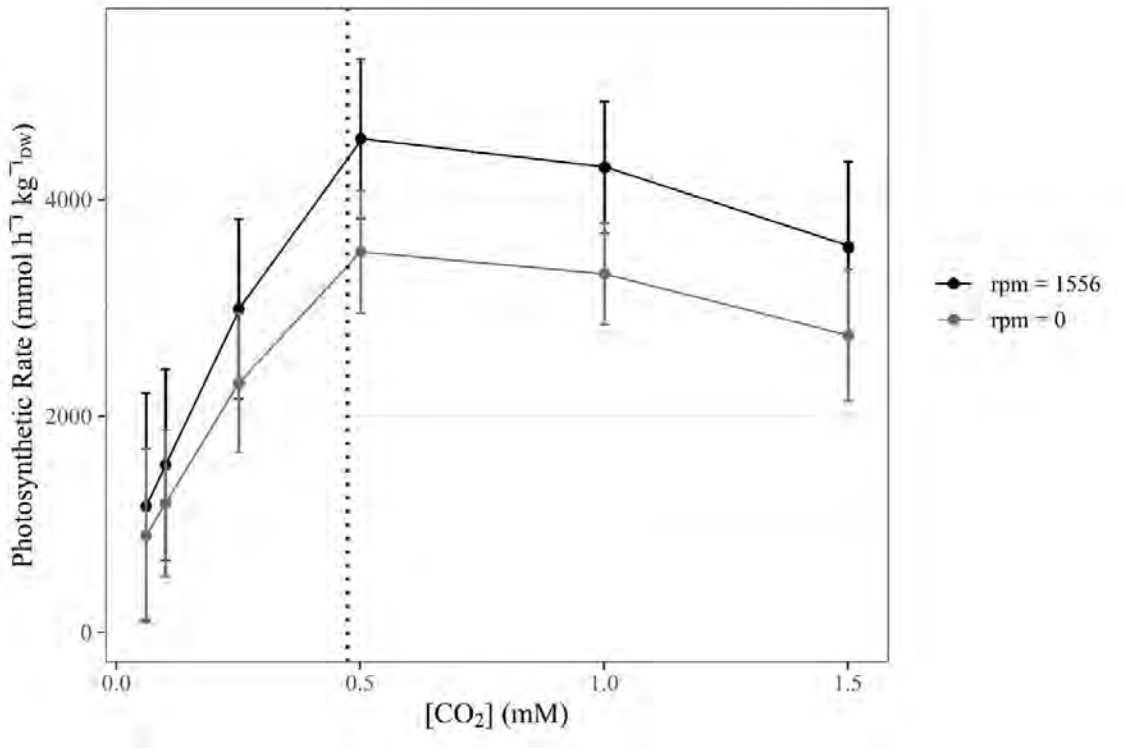

Figure 3.3: CO_2 response curves of *U. stellaris* with "rpm=1556" representing the curve modelled directly from measures taken under highly stirred conditions and the "rpm=0" curve representing the downscaled CO_2 response curve accounting for CO_2 diffusive limitations. The dotted line represents the maximum concentration of dissolved CO_2 recorded in the ambient water of a pond assessed (Chapter 2).

The modelled photosynthetic rates under theoretically well-stirred conditions in situ track the changes in the concentrations of dissolved CO_2 over time (Chapter 2, Figure 3.4), with a maximum photosynthetic rate of 4467.48±319.01 mmol $h^{-1}kg^{-1}_{DW}$ at 08:00. These modelled photosynthetic rates decrease when downscaled for stagnant conditions, with a maximum photosynthetic rate of 3443.89±245.92 mmol $h^{-1}kg^{-1}_{DW}$ at 08:00. This rate is 23% lower than the modelled rate under well-stirred conditions at this time.

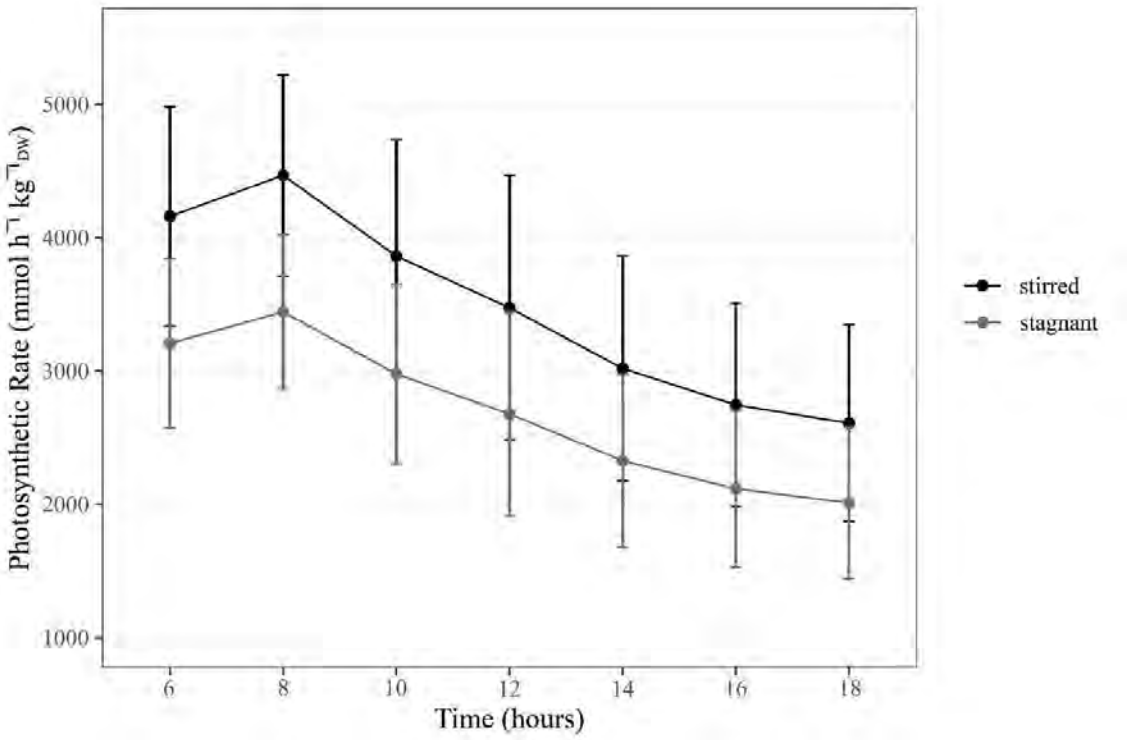

Figure 3.4: Photosynthetic rates of *U. stellaris* modelled at CO_2 concentrations measured throughout daylight hours. The "stirred" curve represents the output from the model where diffusive resistances to CO_2 have not been accounted for, while "stagnant" takes into account these diffusive resistances and their limitations on photosynthetic rate.

Discussion

Photosynthetic rates of *Utricularia stellaris* leaves significantly exceed the photosynthetic rates of both stolon and traps, with trap photosynthetic rates reaching only ±27% of leaves in young plant material and only ±13% in mature plant material. These significantly lower rates of photosynthesis when results were expressed per unit biomass are in accordance with those found in four other species of aquatic *Utricularia* with undifferentiated shoots, as in *U. stellaris* (Adamec, 2006; Knight, 1992). In addition, leaf material makes up approximately 63% of plant biomass per node (Chapter 2). These structures can achieve the greatest photosynthetic rate of all structures and make up majority of plant biomass. Leaf material is, therefore, the primary photosynthetic material of *U. stellaris*. The low rates of photosynthesis of trapping material indicate that these structures are not produced to increase the plant's photosynthetic surface area. The 30% of total biomass invested in these structures (Chapter 2)

is not compensated for by the negligible rates of photosynthesis of traps. Therefore, traps likely serve some other purpose outside of photosynthate production.

The lack of variability in photosynthetic rates between structures, when measurements were standardised per unit chlorophyll, is consistent with measurements in three other species of *Utricularia* with the same growth form (Adamec, 2013). This suggests that differences in photosynthetic rates between structures are likely due to differences in the chlorophyll content of these structures. This is consistent with the chlorophyll content measurements of plant structures (Chapter 2), where chlorophyll *a* and chlorophyll *b* concentrations in leaf material significantly exceed that in trap and stolon material.

Unlike the plant-part photosynthesis measurements, the CO_2 response curves were constructed under standardised conditions similar to those in other studies. The maximal rates of photosynthesis that have been reported in these studies of aquatic *Utricularia* species were found to be approximately 1800 mmol $h^{-1}kg^{-1}_{DW}$ (Adamec, 2013, 2006). The measurements recorded in this study reach a maximum of 4467.48±319.01 mmol $h^{-1}kg^{-1}_{DW}$ at 0.59±0.09 mM CO_2, which is more than double the previous highest documented rate in other species in the genus. The measurements taken here were recorded under slightly different conditions of temperature and CO_2 concentrations, and the plant material used here consisted of whole leaves and not leaves devoid of traps, with the exclusion of the biomass allocated to traps resulting in further inflation of the measured values. The maximal rates of photosynthesis reported here exceed those of aquatic *Utricularia* (Adamec, 2013, 2006) which are known to be comparable to and even higher than the maximal rates of non-carnivorous aquatic species (Adamec, 2018), potentially more so than the deviation that might be expected under the slightly variable measurement conditions. The rates recorded for *U. stellaris* that are on par with the maximum rates of photosynthesis in other aquatic *Utricularia* (Adamec, 2013, 2006) are those measurements taken at low CO_2 concentrations, such as those used in the plant part photosynthesis measurements at approximately 0.02 mM dissolved CO_2. It is known that aquatic carnivorous plants can photosynthesise at a rate that exceeds that of aquatic non-carnivorous plants. However, the rates recorded here are certainly higher than was anticipated.

However, these elevated rates are not representative of the rates that *U. stellaris* individuals will be able to achieve under natural pond conditions. As is evidenced by the in situ photosynthetic model, photosynthetic rates of plants fluctuate substantially throughout the

day due to diel fluctuations in dissolved CO_2 available in the bulk water. At 08:00, respiratory CO_2 produced at night is at a maximum, reaching approximately 0.47 mM dissolved CO_2 in the bulk water. This concentration of CO_2 is almost on par with the point of CO_2 availability where the response curves constructed begin to decrease, the peak measurement at 0.59 mM dissolved CO_2. This peak represents the maximal rate of photosynthesis achievable by *U. stellaris*. Therefore, the photosynthetic rates of plants are largely unlimited in situ at this time based on the CO_2 measurements taken in ponds. Following this time, the photosynthetic rate begins to decline rapidly in response to rapidly consumed CO_2 in the bulk water. Following 08:00 and the pond's exposure to photosynthetically active radiation, plants become CO_2 limited, showing a decrease from maximal rates achievable, to varying extents throughout the day.

However, these levels of limitation do not account for diffusive resistances to the movement of CO_2 through the bulk water and the subsequent deficits of CO_2 concentrations in the boundary layer surrounding leaves. These limitations are clearly illustrated by the fact that the rate of solution stirring in the oxygen electrode chamber substantially affects the photosynthetic rates of plant material, with a decrease in spin rate resulting in a decline in photosynthetic output. Due to all other factors being strictly controlled, this decline is a result of the increasing effect of the CO_2 depleted boundary layer of water surrounding the surface of the plant material. The CO_2 deficit in this layer becomes more pronounced in poorly stirred water. Based on the photosynthetic model showing rates achievable according to dissolved CO_2 in the ambient water, *U. stellaris* is largely unlimited by CO_2 availability at 8:00. However, once one takes into account diffusive resistances, this is no longer found to be the case. Diffusive resistances result in a marked decline in photosynthetic rates, indicating the CO_2 limitation of plants even at the maximum CO_2 concentrations measured at 08:00.

Therefore, even though the dissolved CO_2 concentrations in the bulk water of the *U. stellaris* site assessed (Chapter 2) are very high throughout the day, even after a full day of photosynthetic CO_2 consumption (Chapter 2), and the photosynthetic rate is also, in turn, very high, these rates are still lower than the plant is physiologically able to achieve. This photosynthetic deficit results in a limitation to the amount of energy-rich carbon compounds that, provided CO_2 was not limiting, could allow for increased production of photosynthates, potentially increasing growth rates until another limiting factor is encountered. High concentrations of dissolved CO_2 documented in *Utricularia* habitats are likely to be the

reason why these plants can grow at such a rapid rate (Adamec, 2018), a rate that could potentially be further enhanced were it not for diel CO_2 fluctuations and diffusive resistances.

The biphasic nature of the CO_2 response curves constructed were not that which was expected for a C3 plant. The photosynthetic rate increases steeply and begins to decline once reaching a maximum. It is known that the Calvin-Benson-Bassham cycle is activated by alkalisation of the chloroplast stroma (Flügge and Heldt, 1984; Trinh and Masuda, 2022; Versaw and Harrison, 2002) and that an acidification of the stroma results in downregulation of CO_2 fixation (Maury et al., 1981; Trinh and Masuda, 2022). It is possible that the extremely high CO_2 concentrations in the chamber medium, and resultant high acidification of this medium, resulted in acidification of the stroma, a resultant downregulation of CO_2 fixation and the sudden and consistent decline in photosynthetic rates recorded. However, this remains a theoretical explanation.

It is perhaps reasonable to suppose that *U. stellaris* has likely evolved some mechanism to counter CO_2 limitations and resultant resource allocation to costly photosynthetic physiology that remains partly unused. The leaves of *U. stellaris* are extremely thin and highly branched. This growth form may, in itself, be an adaptation to CO_2 limitation. Maximising plant surface area while minimising the photosynthetic capacity of biomass per unit area is an effective way in which submerged macrophytes can maximise CO_2 uptake while minimising the speed at which these CO_2 sources, the ambient water, become CO_2 depleted (Maberly and Madsen, 2002). Based on this knowledge, it seems that trapping structures should theoretically be photosynthetic, further increasing photosynthetic area, increasing CO_2 uptake and aiding in mitigating CO_2 limitation. However, trapping structures are not photosynthetic and the trap tissue is not contributing to alleviation of CO_2 limitation. These limitations would be alleviated if 30% of total plant vegetative biomass and 30% of total resources were not allocated to non-photosynthetic trapping structures. The production of traps must perform a function that provides benefits to plants that outweigh both their production costs and the cost of photosynthates that could be produced if trapping material resources were allocated to photosynthetic biomass.

Traps could increase fitness in several ways. Based on the “trapping” function of these structures, traps may benefit plants by capturing organisms and detritus suspended in the water column. Trap contents could benefit plants by supplementing the nutrient uptake of these plants either through the carnivorous capture and breakdown of zooplankton prey or

through the nutrient cycling of living mutualistic microbial trap communities (Sirová et al., 2018a).

An additional possibility is that trapping structures may bolster photosynthetic output indirectly, not through the production of photosynthates but as a source of supplementary dissolved CO_2. It is known that dissolved oxygen concentrations within trapping structures are so low that it has been theorised to be the mechanism through which larger prey organisms, unable to withstand such conditions, are killed (Adamec, 2007). These conditions are likely achieved through aerobic respiration of the physiologically active trap water-pumping mechanisms (Adamec, 2007; Płachno and Muravnik, 2018) and the non-photosynthetic component of the living trap microbial community. It is possible that the CO_2 in the internal trap environment is being taken up by the plant and used as an inorganic carbon source for photosynthesis, bypassing the limitations of the boundary layer of water, with CO_2 being produced within enclosed trapping structures. CO_2 supplementation by trap microbes may partly explain the high rates of photosynthesis recorded in this study, where photosynthetic rates of whole leaves, still bearing traps, were used. Perhaps traps were supplementing leaves with CO_2 and supporting photosynthetic rates higher than those recorded in other studies of leaves devoid of traps.

The CO_2 limitations to photosynthetic rate revealed here indicate that, photosynthetic capacity is not at a maximum and, therefore, a portion of the costly photosynthetic physiology produced by plants remains unexploited for majority of the time. Investment in this physiology may be a means to make use of CO_2 whenever it does become available. However, dissolved CO_2 concentrations measured in a pond of *U. stellaris* (Chapter 2) were in the range of the highest CO_2 concentrations measured in freshwater systems. CO_2 concentrations are unlikely to be elevated further in ambient water. CO_2 concentrations may be elevated further by supplementation of CO_2 from the inner trap environment, allowing for plants to utilise their full photosynthetic potential, ensuring that the production of costly photosynthetic physiology is not causing an additional cost through lack of use.

Another potentially relevant trap trait is the change in trap wall colouration from transparent green to a largely opaque trap wall, with the accumulation of purple anthocyanin pigments (Chapter 2(Sirová et al., 2018a)). These pigments influence the intensity and quality of light reaching the trap lumen, influencing the photosynthetic activity of autotrophic microbes (Sirová et al., 2018). Perhaps this change in light is a means to bolster the respiratory output

and CO_2 production of accumulated living microbial communities. This would provide the plant with additional CO_2 before these trapping structures reach senescence and are lost. However, these lines of thought are merely speculative and require further investigation.

Conclusion

U. stellaris is CO_2 limited in situ due to both diel CO_2 fluctuations and CO_2 diffusive resistances. This limitation is compounded by the fact that plants allocate resources to producing non-photosynthetic traps. Therefore, traps must provide a benefit that outweighs both the costs of trap production and maintenance and the costs of the photosynthates that could have been produced were these resources allocated to photosynthetic leaves. Based on the trapping capabilities of these structures, benefits could potentially be derived from trap contents, perhaps for their ability to supplement plants with respiratory CO_2, nutrients or a combination of the two.

Chapter 4: The use of trapping structures for the housing of living microbial communities in the "carnivorous" plant *Utricularia stellaris* L. fil. (Lentibulariaceae)

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Introduction

The definition of botanical carnivory (Ellison et al., 2018) states that for a plant to be considered carnivorous, it needs to have evolved the ability to capture prey in specialised traps. Plants that have evolved such structures gain mineral nutrition from captured prey (Ellison et al., 2018), allowing for these plants to gain a competitive advantage over co-existing non-carnivorous plants in nutrient-poor habitats (Givnish et al., 2018, 1984). These plants vary in terms of the ranges of prey types captured (Ellison and Gotelli, 2009), differences that are generally a result of plant species occurring in different habitats characterised by different assemblages of prey organisms, as well as the differences in morphological specialisation in trapping structures between genera (Ellison and Gotelli, 2009).

A group of carnivorous plants in which trap contents are of particular interest are the aquatic species of the genus *Utricularia*. This genus of plants has one of the most complex trapping mechanisms of all carnivorous plants (Juniper et al., 1989), utilising minute suction traps. These traps are used to capture prey organisms such as Copepods, Cladocerans, insect larvae and other microinvertebrates (Alkhalaf et al., 2009; Płachno et al., 2012; Richards, 2001) and the prey captured by aquatic *Utricularia* is usually dominated by Cladocera and cyclopoid Copepods (Ellison and Gotelli, 2009).

Despite the capture of these prey organisms having been documented, prey capture rates in aquatic *Utricularia* are frequently low, with less than 50% of the traps of *U. australis* and *U. vulgaris* (Alkhalaf et al., 2009) and less than 10% of the traps assessed in *U. purpurea* containing expired prey organisms (Richards, 2001). This low capture rate is unsurprising, as aquatic *Utricularia* grow in nutrient-poor environments, where zooplankton are often scarce (Bern, 1997). Counter-intuitively, there is generally a significant investment in traps in

aquatic *Utricularia* species (Richards, 2001) despite low prey-capture rates. It is possible that even if 10% of a plant's traps captured prey organisms, this could be sufficient to meet the nutrient requirements of a plant. However, in *U. foliosa*, establishment of a nutrient budget indicated that carnivory provided plants with less than 1% of the nitrogen and less than 2% of the total phosphorus required by the plant (Bern, 1997). The low levels of prey capture and resultant limited nutrient gain from prey suggest that benefits derived from carnivory may be too low to compensate for the heavy investment in traps.

While many trapping structures lack zooplankton prey, these traps do not remain unoccupied. In studies reporting low zooplankton capture rates, almost all mature traps contain living communities of microorganisms, consisting primarily of bacteria, algae, protists, microfungi, rotifers, and associated detritus (Richards, 2001; Sirová et al., 2018a, 2009). Although the trap microbial community is primarily derived from the external trap periphyton community, some organisms are unique to traps, including Desmidiales and Euglenophyceae, which may prefer the unique nutrient and pH conditions within trapping structures (Sirová et al., 2009). In addition, the microbial biomass within the traps of *U. purpurea* increased with trap age (Sirová et al., 2009). This illustrates that these communities are living within traps and that this environment harbours a unique, specially adapted microbial assemblage.

Perhaps aquatic *Utricularia* species gain benefits derived from living microbial trap communities, a theory which has brought into question the importance of carnivory in aquatic *Utricularia* species (Richards, 2001). These microbial communities are unique from those found in the traps of other carnivorous plants, with the trap microbes of two aquatic *Utricularia* species having been found to contain a far greater diversity of prokaryotes compared to the traps of other carnivorous plants (Sirová et al., 2018b). While the benefits of microbial communities within the traps of other carnivorous plant species mainly surround the process of prey digestion (Bittleston, 2018), it is possible that, in the complete absence of prey organisms, microbial communities take on other beneficial functions.

Despite trap contents having been documented in representative species of aquatic *Utricularia*, an assessment of the trap contents and prey trapping frequency in *U. stellaris*, an understudied species, has not been undertaken. Due to this lack of study, it remains unresolved whether prey capture rates in this species are low enough to indicate that the possibility of benefits being derived from living microbial communities demands further investigation.

Aims and hypotheses

As in the findings of Alkhalaf et al. (2009) and Richards (2001), preliminary surveys of fresh traps of *U. stellaris* indicated that living microbes are abundant throughout traps, while few traps contain expired zooplankton prey. Therefore, I hypothesised that the proportion of traps of *U. stellaris* containing living microbial communities are ubiquitous and far exceed those containing zooplankton prey. To investigate this hypothesis, a survey of the trap contents of *U. stellaris* will be conducted. If the majority of traps are lacking in zooplankton prey but do contain microbial communities, then benefits may be derived from these microbes.

The observation that the microbial communities are alive within the trap environment led to the hypothesis that these microbes may form stable, successful communities within traps of *U. stellaris*. A preliminary assessment of the trap microbial communities will be conducted to assess this community's size and status with increasing age.

If the proportion of traps containing living microbial communities far exceeds the proportion containing prey, *U. stellaris* may maintain a high biomass investment in traps due to the benefits gained from these ubiquitous living communities. These results would indicate that the potential benefits derived from these communities should be investigated further.

Methods and Materials

Trap content assessment

U. stellaris individuals assessed were collected on the 29th and 30th April 2021 at sites 1(a), 2(a) and 3(a) (Appendix A). All samples were returned to the lab, where they were stored in the dark at 8°C and processed within four days following collection (Richards, 2001). One leaf at every fourth node was used to assess trap contents. At the time of collection and processing, leaves from successive nodes were excised and placed in a microcentrifuge tube of distilled water. The leaf was subjected to 3 minutes of manual shaking to remove periphyton from the leaf's surface, after which this periphyton solution was discarded. The leaf was then placed in a microcentrifuge tube of FAA preservative for later inspection. Leaves were observed under an Olympus SZH-ILLD stereomicroscope to assess trap contents. Trap contents were observed by focusing the microscope through the trap walls onto the contents, which was possible due to the very thin, relatively transparent trap walls. The total number of traps per leaf and the number of traps containing zooplankton were counted.

The presence of detritus was used as an indication of the presence of a microbial community within the trap. The trap detritus consisted of an indistinguishable mass of fine, brown, organic matter that, in freshly collected (unpreserved) traps, was fed on extensively by a large community of living ciliates and other microbes. The number of traps per leaf containing this detritus were counted.

Microbial community assessment

The trap algal community was then assessed. The algal community was treated as a proxy for the overall microbial community, which typically includes living bacteria, protozoa, and rotifers in addition to algae. Algae, observed to be living on initial assessment of freshly collected (unpreserved) traps, were relatively large, easily visible, reliably preserved and provided an indication of the condition of the microbial community as a whole. The four largest traps along the length of every preserved leaf were excised. Each trap was broken open on a microscope slide, and all trap contents were removed. A coverslip was placed over the contents, and the microscope slide was viewed under an Olympus CX21LED light microscope. The length of the coverslip was divided into five points, each representing a field of view under the microscope at 100X magnification. Within each of these fields of view, all organisms were identified to morphospecies and counted to assess the abundance of algae within traps and the algal species richness in traps, species richness being the total number of taxonomic units making up a community (Magurran, 2021).

Statistical analysis

The proportion of traps per node "with zooplankton" and "without zooplankton" as well as "with algae" and "without algae" were contrasted between nodes using a generalised linear mixed-effects model (GLMM) with a beta-binomial error distribution and a logit link function, due to overdispersion of both the zooplankton and algae datasets. Initially, the model could not run due to 0% of traps at node 4 containing zooplankton and 100% at nodes 20 and 28 containing algae. To overcome this issue, mock data was inputted. For the node 4 dataset, one "0" value was replaced with a "1", and for the node 20 and 28 datasets, a value of "1" was subtracted from a single count at each of these nodes. The site was treated as a random factor in these models. Due to the overdispersion in the response variables "algal abundance" and "algal species richness," a generalised linear model (GLM) with a negative binomial error distribution and log link function was applied to both datasets. However, both negative binomial models still showed overdispersion. Due to this overdispersion and the

high frequency of zero observations, zero-inflated Poisson models were used. The site was treated as a random factor in these models. All GLMMs and zero-inflated models were calculated using the glmmTMB command from the package "glmmTMB" (Brooks et al., 2017). ANOVAs were completed for each model using the Anova command from the package "car" (Fox and Weisberg, 2019). Estimated marginal means were obtained using the emmeans command from the package "emmeans" (Lenth, 2023). All statistical analyses were conducted in R (R Core Team, 2023).

Results

Trap content assessment

There was no significant difference between the proportion of traps per leaf containing zooplankton between successive nodes ($P>0.05$). Additionally, there was only a significant difference in the proportion of traps occupied with algal communities between nodes 4 and 8 ($P=0.0006$), representing the period during which traps primarily become occupied by algae. After node 8, there are no significant differences in the proportion of traps occupied by algae. Even though there are few differences between the proportion of traps containing either zooplankton or algae between nodes, it is clear that substantially more traps per node contain algae as opposed to zooplankton (Figure 4.1), with the maximum proportion of traps containing zooplankton being 34.56± 9.86%. In comparison, algal trap occupation approaches 100%, with algae being present in all traps on a leaf.

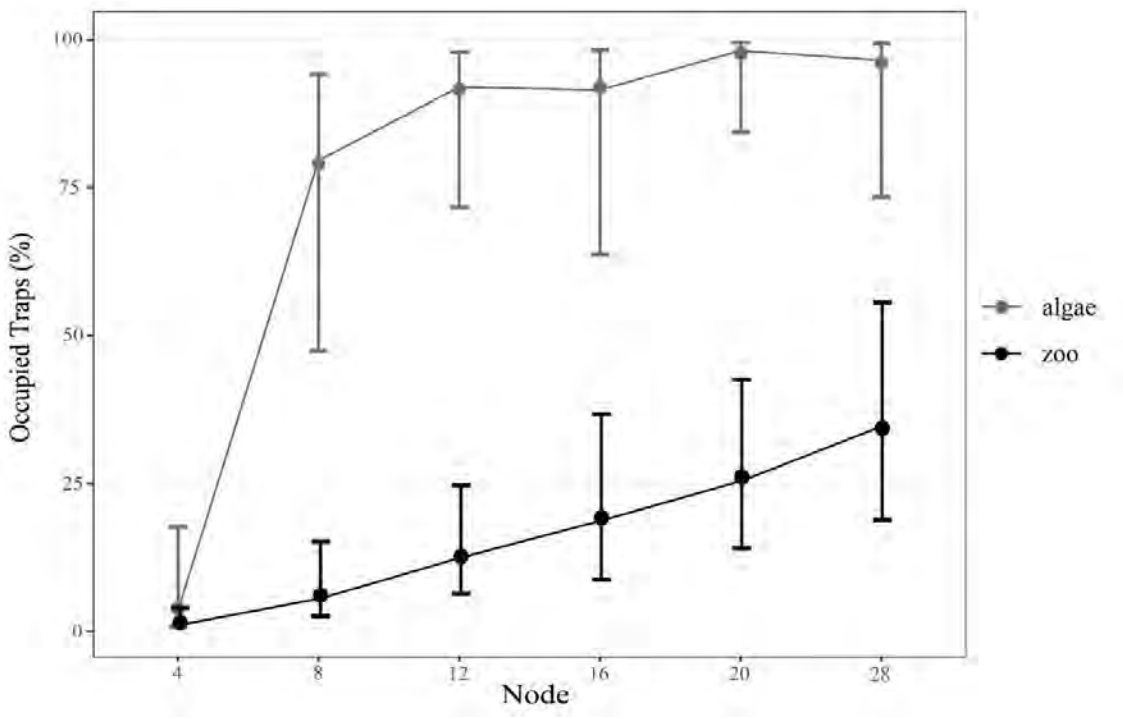

Figure 4.1: The percentage of traps per leaf occupied with zooplankton (black) and algae (grey) at successive nodes along the length of the stolon.

Copepods are the most common type of prey organism encountered in the traps of *U. stellaris*. These are followed by the less frequently encountered Chironomid larvae, Nematodes, water mites in the order Trombidiformes and Cladocerans in the families Daphniidae and Chydoridae. Rarely encountered prey organisms include Ephemeroptera and Trichoptera larvae and, encountered only once, a small damselfly larva (Odonata).

Certain algal representatives were identified to a level more precise than morphospecies. Of these, the most commonly encountered genera in the traps are *U. stellaris* visible under 100X magnification include *Closterium*, *Gonatozygon*, and *Microcystis* and *Botryococcus* colonies. Less frequently encountered algae include *Pleurosigma, Pinnularia, Cosmarium, Peridinium* and *Euglena. Volvox* colonies were rarely encountered, only recorded a total of three times.

Observations of freshly collected (unpreserved) traps revealed living microorganisms other than algae, with a large population of ciliates and rotifers in evidence.

Microbial community assessment

Node number significantly affected the algal species richness of traps (χ^2=52.479; df=6; P<0.0001). Algal species richness increases significantly between nodes 8 and 12 (P=0.0498) (Figure 4.2). Additionally, node number significantly affected the abundance of algae per trap (χ^2=1712.29; df=6; P<0.0001). Pairwise comparisons show that algal abundance per trap declines significantly between nodes 4 and 8 (P<0.0001) but increases significantly between nodes 8 and 12 (P<0.0001) and nodes 12 and 16 (P<0.0001) (Figure 4.2). Algal abundance remains stable from node 16 until node 28 (P>0.05), after which algal abundance undergoes a further increase between nodes 28 and 36 (P=0.0031).

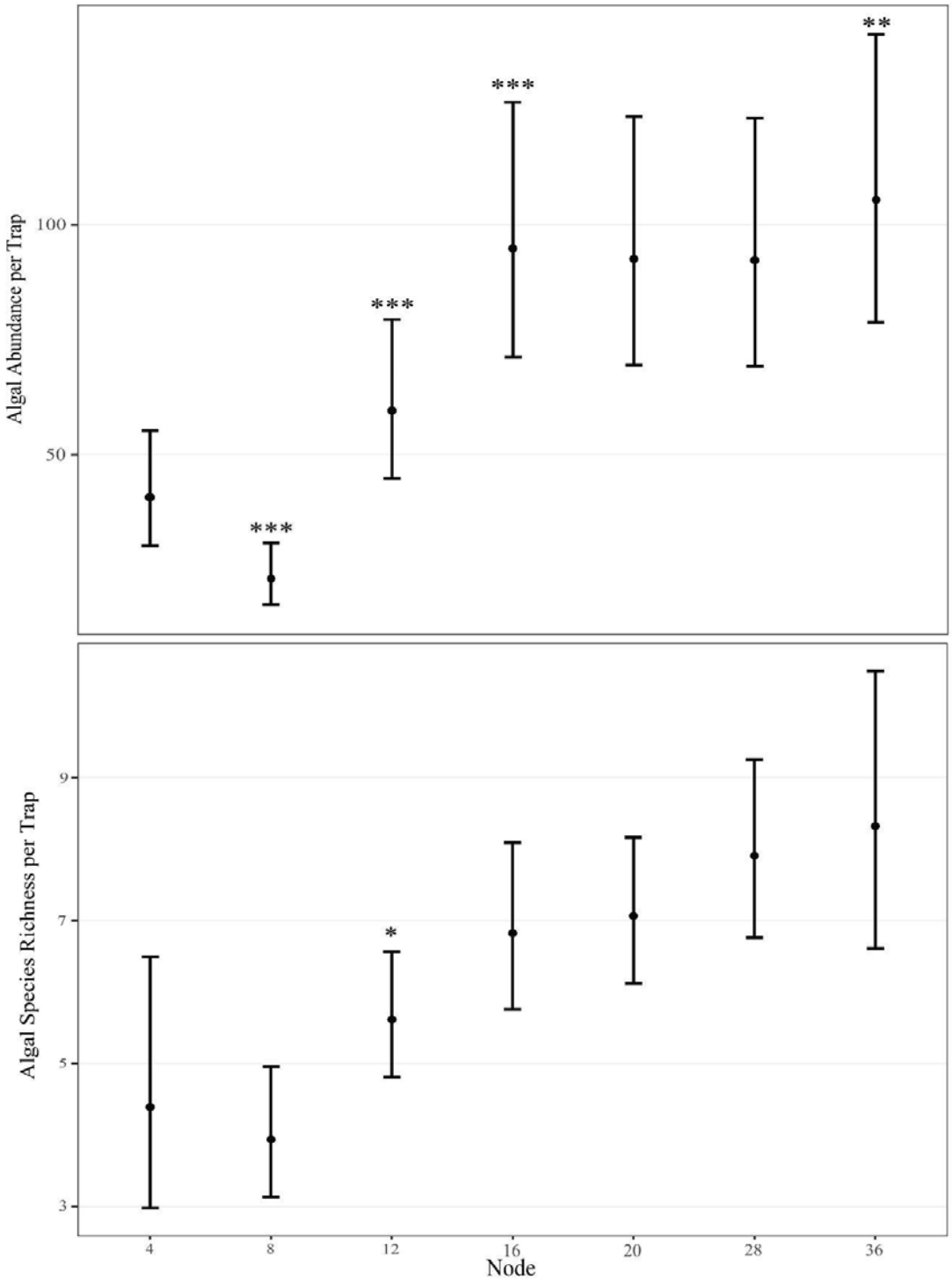

Figure 4.2: The average species richness per trap and the average algal abundance per trap for successive nodes along the length of the stolon. Asterisks indicate significant differences between successive nodes along the length of the stolon: ***: P<0.001; **: P<0.01; *: P<0.05.

Discussion

Trap content assessment

In aquatic *Utricularia* species, prey organisms are distinguished from those forming the microbial community according to which organisms can survive the internal trap anoxia (Adamec, 2007) for a prolonged period and are able to reproduce in this environment (Sirová et al., 2018a). Larger organisms that cannot survive under oxygen concentrations of less than 1 μM are killed. Assessment of trap contents in *U. stellaris* reveals that, from the onset of trap functionality (Chapter 2), the proportion of traps containing algae and, therefore, living microbial communities undergoes a substantial increase, with over 75% of traps colonised by microbes by node 8. This rapid colonisation of traps is possible due to the ease with which these small organisms are passively dispersed and aspirated into traps during firing events (Finlay and Clarke, 1999), and the organic plant exudates in traps initially bolstering these communities (Sirová et al., 2018a). Based on the ubiquity of microbes within traps, one might expect that similar proportions of traps would contain expired zooplankton prey. However, this is not the case. Throughout nodes, substantially fewer traps contain zooplankton compared to microbes, with just less than 35% of traps containing expired zooplankton prey. The ubiquity of microbial communities and scarcity of zooplankton prey throughout *U. stellaris* traps has also been documented in other species of aquatic *Utricularia* (Alkhalaf et al., 2009; Richards, 2001). Despite low zooplankton trapping rates, it is possible that the quantity of zooplankton captured is sufficient to meet the nutritional requirements of *U. stellaris*. This would explain why trap production is maintained despite what appears to be low zooplankton capture. However, based on the findings of Bern (1997), it seems that nutrition gained from zooplankton prey contributes a negligible amount of total required plant nutrition, supporting the hypothesis that additional nutrients are being gained elsewhere, perhaps from living microbial communities which are abundant and ubiquitous throughout traps.

As traps mature, algal abundance within traps levels off and stabilises. This stability in abundance may seem counterintuitive when one considers that trap functionality undergoes a concomitant decline (Chapter 2). If microbial organisms were being subjected to carnivory by the plant and microbes, too, have been captured for the sole purpose of being broken down for their component nutrients, the number of organisms in a trap would decline with the decline in trap functionality, as these communities are no longer being augmented with algae

aspirated into traps from the surrounding environment. However, this is not the case. Microbial communities remain stable despite a decrease in trap functionality and even undergo an increase in abundance, indicating that these microbes are alive and successful enough within traps to form a stable community. Even if these microbes eventually expire due to the natural course of senescence, they do not expire due to capture, which does not align with the definition of botanical carnivory (Ellison et al., 2018).

Therefore, based on the uniquity of living microbial communities in the traps of *U. stellaris*, it is possible that plants may be gaining some benefit from these communities which warrant further investigation.

Possible benefits derived from microbial trap communities: Nutrient Derivation

Based on the environmental assessments of *U. stellaris* habitats (Chapters 2 and 3), the primary limiting factors to the productivity achievable by *U. stellaris* are low availability of nutrients and dissolved carbon dioxide.

Living microbial communities likely aid in the digestion of and nutrient liberation from prey organisms (Sirová et al., 2018a, 2009, 2003). However, it is possible that microbial communities can provide plants with nutrients regardless of whether larger prey organisms are captured. High levels of nutrients have been documented to be available within traps of other aquatic *Utricularia* species, whose sole contents consist of living microbial communities (Sirová et al., 2009). This suggests that these microbial communities are responsible for this high level of available nutrients. These nutrients are likely made available from the microbial mineralisation and hydrolysation of complex organic matter, liberating nitrogen and phosphorus from organic detritus aspirated into traps during firing events (Sirová et al., 2018a). The nutrients liberated by these communities would be solely available to the housing *Utricularia* and entirely unavailable to competitors, allowing *Utricularia* to dominate their challenging low-nutrient niche.

Possible benefits derived from microbial trap communities: CO_2 supplementation

It is possible that microbial communities not only increase the availability of nutrients, but the heterotrophic component of the community may also act as a source of carbon dioxide to the plants, which may be in short supply in the shallow, lentic ponds in which *Utricularia* grow (Falkowski and Raven, 2013).

In aquatic systems where inorganic carbon availability is often limiting, some rooted plants are able to increase their CO_2 uptake from sediments in which CO_2 concentrations in the interstitial water can be exceptionally high due to microbial mineralisation (Pedersen et al., 1995). *Utricularia stellaris* lacks roots and is not in contact with the substrate. However, it is possible that similar microbial activity to that in sediments could be taking place in traps. This microbial activity may increase the concentrations of CO_2 within the trapping environment, potentially supplementing *Utricularia* and support higher photosynthetic rates than predicted based on the CO_2 available in the ambient water. However, this possibility is merely theoretical, remains unexplored in *Utricularia stellaris* and requires further study.

Possible benefits derived from being housed in traps of aquatic Utricularia

The ability of algae to maintain a stable population within traps suggests that this community is successful within traps, and it is not inconceivable that these communities may even benefit from being housed in traps.

In aquatic systems, macrophytes often harbour diverse microbial populations adapted to the unique niche created by these plants. The roots of macrophytes are often heavily externally colonised by microbes, and many aquatic plants have been documented to exude nutrients, photosynthates and oxygen as a means to support this community (Stottmeister et al., 2003). These microbial communities can provide plants with otherwise inaccessible nutrients and defence against pathogens (Stottmeister et al., 2003). The theory that plants may support microbial trap communities is supported by results showing that two aquatic *Utricularia* species exude organic substances produced through photosynthesis into the trap environment (Sirová et al., 2010). In addition to exudates, microbial trap communities may simply benefit from the discrete environment created by traps in which organisms can easily interact and products of biological activity can easily be exchanged.

Therefore, it is not inconceivable that aquatic *Utricularia* and trap microbes may influence one another to a mutually beneficial end (Bronstein, 2015). However, the potential plant-microbe benefits are wholly unexplored in *Utricularia stellaris* and remain unconfirmed in other aquatic *Utricularia* species, demanding further study.

Conclusion

Based on the ubiquity of microbial trap communities, the scarcity of zooplankton prey and the proclivity of plants to maintain a high level of investment in trapping structures, plants are likely to gain some benefit from trap microbial communities. In addition, the stability of these communities and the documented exudation of photosynthates into traps in other aquatic *Utricularia* species (Sirová et al., 2010) are indicative of microbial communities gaining a benefit from being housed. If these benefits are confirmed, this interaction would constitute a mutualism, being a mutually beneficial, interspecific interaction (Bronstein, 2015). However, confirmation of interactions requires further study.

Chapter 5: General Discussion

Introduction

The carnivorous syndrome arose within the Lentibulariaceae three times, resulting in three genera with contrasting trapping mechanisms. Traps within the family are highly variable, including the simple flypaper traps of *Pinguicula*, eel traps of *Genlisea*, and *Utricularia's* complex suction traps (Ellison and Gotelli, 2009; Fleischmann et al., 2018). While these highly active *Utricularia* traps have been traditionally interpreted to be used by the plant for carnivory, studies have shown remarkably low trapping rates of zooplankton prey as opposed to the high proportion of traps that contain living communities of microorganisms (Alkhalaf et al., 2009; Richards, 2001). Perhaps the ubiquitous presence of these microorganisms is indicative of benefits being derived from these organisms. However, the role of trap microorganisms in the success of aquatic *Utricularia* species remains unresolved.

To date, little is known about African species of *Utricularia*, including *Utricularia stellaris*, which is widespread in the Afro-Asian tropics (Taylor, 1989). Therefore, this thesis aimed to come to document aspects of the environmental, growth and physiological characteristics of this species to inform future research into the trap-microbe interaction.

Summary of findings

I began by documenting the environmental characteristics prominent in the habitats of *U. stellaris* to reveal what stressors may be driving adaptation in these plants. During this study, the water conditions in 15 sites of *Utricularia stellaris* were assessed, with primary macronutrients, water physicochemical parameters, and diel fluxes in gas availability being recorded. These small, shallow, stagnant ponds were found to be deficient in both the macronutrients nitrogen and phosphorus. However, ponds remained unexpectedly productive, as was evidenced by the measured diel fluctuations in dissolved gases driven by high levels of photosynthesis and respiration. As in the habitats of other aquatic *Utricularia* species (Adamec, 2009), dissolved CO_2 concentrations in the ambient water were high. Based on these results, the primary stressor being experienced by these plants is nutrient limitation, as is characteristic of the habitats in which carnivorous plants are found to grow (Givnish et al., 1984).

I then proceeded to assess the morphology and developmental patterns of *U. stellaris* to identify potential adaptive traits these plants may have evolved for survival in light of the environmental stressors they are subject to. *Utricularia stellaris* morphological adaptive traits, these plants being free-floating, with thin, finely branched leaves, allowing for maximal gaseous exchange with the ambient water and exposure to light and minimal self-shading (Reut et al., 2021). In addition, the substantial biomass allocation to the production of traps, despite their narrow window of functionality, suggests that these structures are likely benefiting plants in some way that outweighs the costs of their production. Based on the environmental factors assessed, these benefits could aid in overcoming nutrient limitations which would allow plants to maintain the high levels of productivity characteristic of aquatic *Utricularia* species (Friday, 1989). The complex trapping function of these structures (Juniper et al., 1989) and the attractive nature of appendages surrounding trap doors (Meyers and Strickler, 1979) suggest that traps may be important to plants for their captured contents.

Despite an initial investigation revealing high CO_2 concentrations in the ambient water of the habitats of *Utricularia stellaris*, knowledge surrounding CO_2 limitations to photosynthesis in aquatic habitats (Black et al., 1981; Smith and Walker, 1980) necessitated further investigation into the effects of this gas on photosynthetic rates achievable. The photosynthetic rates of traps, leaves and stolon material were measured, and it was found that leaves are the primary sites of photosynthesis. The contribution of trap tissue to photosynthate production was negligible, a further indication that traps may be beneficial to plants for their contents. In addition, photosynthetic rates in situ are limited by both diel fluctuations in ambient CO_2 concentrations and limitations due to diffusive resistances opposing CO_2 diffusion through water. These results indicate that *U. stellaris* is subject to not only nutrient limitations but also limitations to photosynthetic rates due to CO_2 deprivation.

As noted, the complex function of traps and the negligible contribution of trap tissue to photosynthate production indicate that traps are likely beneficial for their ability to capture and house what is taken up from the water column. Therefore, the trap contents of *Utricularia stellaris* were assessed. The proportion of trapping structures that contain living microbial communities far exceeds the portion that contains larger zooplankton prey. Despite these low prey capture rates, investment in trapping structures remains high, indicating that ubiquitous microbial communities may benefit plants. In addition, these communities are stable and self-sustaining, indicating their health and the possibility that they, too, benefit from their interaction with *U. stellaris*. The low zooplankton trapping rates and ubiquity of

living microbial trap communities indicate that further investigation into the plant-microbe interaction in *U. stellaris* is required.

Thus, the results of this thesis highlight the unique conditions that characterise aquatic habitats, such as CO_2 deficits limiting photosynthetic rates. This stressor, combined with the importance and ubiquity of microbial nutrient cycling, may have caused traps to take on an additional role in aquatic habitats, housing microbial communities beneficial in prey-free traps, in addition to the carnivorous capture of prey. Prior to this, the ecology of *U. stellaris* has been largely overlooked. The results of this thesis provide a useful framework which will inform future investigation into the plant-microbe interaction.

Future directions

The benefits and relative contributions of subsets of trap contents, zooplankton prey and microbial communities, remain unresolved. *Utricularia* likely gains a benefit from microbial trap communities, and assessments of trap community stability suggest that microbial communities are also gaining a benefit from being housed, with the exudation of photosynthates into trap environments having been documented (Sirová et al., 2010). In ecological studies, nutrient transfer between organisms can be revealed by using stable isotope tracing (Feldhaar et al., 2009; Reichardt and Timm, 2020). Aquatic *Utricularia* are likely mutually beneficial to one another, with plants providing photosynthates to microbes and trap microbes making nitrogen and phosphorous available through the mineralisation of detritus. To elucidate whether the plant takes up nutrients from the trap and which sources provide these molecules, isotopic tracing will be employed to definitively illustrate the uptake of contents of trapping structures and distribution of that which is taken up throughout plant tissues. This will allow one to determine whether nutrients or inorganic carbon are being taken up by plants from the trapping environment and whether these molecules are derived from zooplankton prey or living microbial communities.

Another factor that remains unresolved following this thesis and in aquatic *Utricularia* species in general (Adamec et al., 2010) is consistent and reliable evidence supporting the use of metabolites gained through carnivory for plant growth and development. Benefits derived from the plant through a potential mutualism will likely be reflected in an increase in vegetative growth or reproductive output, which are often challenging to uncouple in aquatic plants, with their high affinity for clonal reproduction. Despite the importance of clonal

reproduction in these plants, seed production in aquatic habitats should not be overlooked. It has been observed that, in *U. stellaris*, increased production of inflorescences is often paired with a decline in the vegetative vigour of plants. This trend is well established in carnivorous plants, with investment in reproductive structures shown to result in a proportional decrease in the production of vegetative plant parts and the maintenance of these structures (Southwick, 1984). To fully understand whether and how the production and occupation of trapping structures benefit a plant, it is necessary to elucidate which facets of plant growth reflect these benefits: increasing vegetative growth and clonal reproduction, an increased investment in inflorescence production or a combination of the two. Plant vigour and clonality can easily be quantified. However, not only does the number of flowers produced play a role in the quantity and quality of the seed set, but also the success with which flowers are pollinated. The floral biology of carnivorous plants has been widely overlooked, with the mechanisms behind their unusual nutrient supplementation being the key point of interest. The genus *Utricularia* falls into this understudied group, with only a handful of the 214 described species having had any investigation into their reproductive biology (Taylor, 1989). Of those that have had comprehensive studies conducted, almost all species are self-compatible but not autonomous-selfers; the flower morphology of most of the genus demands that a pollinator mediate pollen transfer (Hobbhahn et al., 2006). Therefore, pollinator availability, attraction, and fit likely play an important role in determining the importance of investing in costly reproductive structures, expressed as seed set and quality.

To investigate the link between trap-mediated nutrient supplementation and plant vigour, plant growth will be correlated to the abiotic conditions of ponds. This will indicate how plants respond to an environmental stressor, potentially investing more in trapping structures in response to low nutrient or CO_2 availability. Following this, plant vigour will subsequently be correlated with the reproductive output of plants to understand the links between environmental stressors, plant vigour and plant reproductive output. To achieve this, a breeding system of *U. stellaris* will be established to understand what portion of the reproductive output is due to plant vigour and what portion is attributable to the action of pollinators.

This future research will aid in further understanding the functions of trapping structures in *U. stellaris*, determining whether traps are primarily beneficial for housing microbes or capturing prey. In addition, it will aid in clarifying the link between uptake from

trapping structures and the use of these metabolites by plants to increase vigour or reproductive output, addressing an ambiguous topic throughout aquatic *Utricularia* species.

Conclusions

Utricularia stellaris is an aquatic macrophyte which produces complex suction-trapping structures (Juniper et al., 1989). These structures have traditionally been interpreted to be important to plants for facilitating carnivory, capturing zooplankton prey present in the water column. However, most of these traps lack zooplankton prey, containing only living communities of microorganisms. Despite this lack of prey, biomass allocation and resource allocation to these structures remain high, suggesting that the ubiquitous microbial communities may benefit plants in the absence of prey.

U. stellaris is subject to two major limitations in ponds: nutrient limitation and CO_2 limitation. Besides several morphological adaptations to survival as a submerged aquatic plant, the high biomass allocation to the production of traps indicates that traps are likely an adaptation to enhance survival. The tissue of the trapping structures does not contribute substantially to photosynthate production, but based on their complex functions specialised for trapping that which is suspended in the water column, it is probable that any benefits derived from traps are sourced from their contents.

Assessment of trap contents and the ubiquity of trap microbial communities suggests that benefits may be derived from the metabolism of trap microbes in addition to benefits of prey capture. However, quantification of such benefits requires further study.

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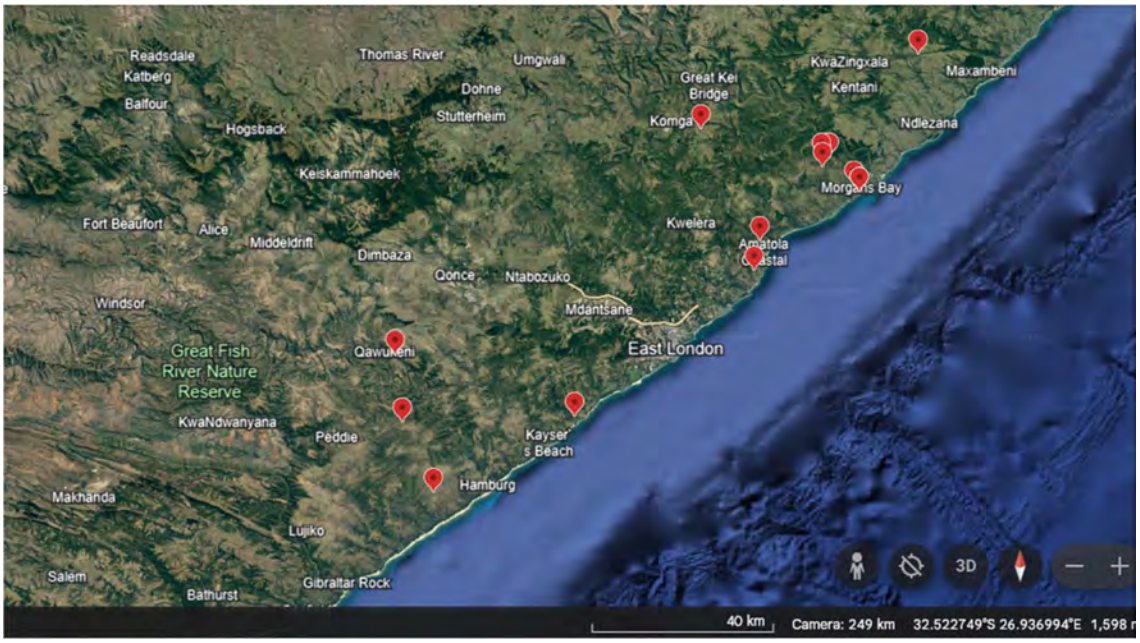
Appendix

Appendix A: All the sites of *U. stellaris* used in this study, including the date and time of assessment of those used for water condition assessment (sites 1-15) (Chapter 2)

Site	Locality	Coordinates		Date	Time
1	Wesley	33.305344°S	27.342465°E	11-Mar-23	13:38
2	Cross Roads	33.167874°S	27.269490°E	11-Mar-23	16:32
3	Kidd's Beach	33.156201°S	27.671520°E	08-May-22	16:23
4	Qawukeni	33.035266°S	27.252895°E	20-Jun-22	15:00
5	Queensberry Bay	32.869655°S	28.088704°E	05-Apr-23	15:30
6	Cintsa	32.811256°S	28.101213°E	05-Apr-23	14:11
7	Morgan Bay cliffs (1)	32.712862°S	28.333138°E	19-Jun-22	12:00
8	Morgan Bay cliffs (2)	32.712862°S	28.333138°E	19-Jun-22	12:00
9	Morgan Bay	32.700386°S	28.320001°E	07-May-22	13:57
10	Morganville	32.665125°S	28.245723°E	20-Jun-22	07:30
11	Mkulu Kei (1)	32.647111°S	28.242972°E	06-May-22	12:50
12	Mkulu Kei (2)	32.647111°S	28.242972°E	19-Jun-22	11:00
13	Lalapanzi	32.646088°S	28.261014°E	07-May-22	14:50
14	Komga	32.593800°S	27.960620°E	08-May-22	14:47
15	Kabakazi	32.441967°S	28.466611°E	18-Jun-22	13:13
1(a)	Mkulu Kei	32.647111°S	28.242972°E		
2(a)	Kei Mouth Reservoir	32.697624°S	28.349853°E		
3(a)	Haga Haga	32.713889°S	28.178889°E		



Appendix B: A map showing sites one to fifteen