Low atmospheric CO_2 induces nocturnal carbon accumulation in the lycophyte genus *Isoëtes*

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Abstract

Metabolic shifts play an essential role in the survival of plants in extreme habitats. Certain desert plant species, for example, avoid water loss by temporally segregating the light and dark reactions of photosynthesis. By only opening their stomata at night these plants inhibit significant water loss during the day. At night CO_2 is incorporate into 4-carbon acids for subsequent daytime fixation[1-5]. This behavior is known as Crassulacean Acid Metabolism (CAM) and leads to noticeable diel cycles in pH of photosynthetic organs[1-5]. Oddly, similar acidity cycles are found in the submerged aquatic lycophyte genus *Isoëtes*[2,6-12], which are not water limited in any sense. It has long been assumed that their nocturnal CO_2 accumulation is an adaptation to low daytime carbon levels in aquatic ecosystems, but this has never been empirically tested[2,6-13]. Here, we offer direct evidence that CO_2 starvation induces CAM-like nocturnal carbon accumulation in terrestrial *Isoëtes*. Populations of terrestrial *Isoëtes engelmannii* grown in climate-controlled chambers and starved of atmospheric CO_2 during the day displayed diel acidity cycles similar to those in both xerophytic CAM plants and submerged *Isoëtes* species. These results substantiate the hypothesis that carbon starvation provides a selective pressure for nocturnal carbon accumulation in *Isoëtes*. Furthermore, while aquatic CO_2 levels undoubtedly promote nocturnal carbon accumulation in extant *Isoëtes*, the induction of this behavior in *terrestrial* plants suggests a possible earlier terrestrial evolution of this metabolism in response to low *atmospheric* CO_2 levels during the Carboniferous period[14-17]. Our findings therefore both provide empirical support for a long-standing assumption about nocturnal carbon accumulation in the lycophyte lineage[2,6-12,18] and suggest an earlier evolution of this behavior, leading to the notion that CAM in xerophytes may only represent a subset of metabolisms that employ nocturnal carbon accumulation in response to variable environmental pressures.

Plants inhabit some of the harshest terrestrial environments on earth. In deserts the metabolic pathway known CAM evolved in some lineages to prevent dessication[1-5,13,18]. This metabolism is critical for the success of many xerophytic plants and has been regarded as one of the most important adaptations to dry environments[1-5]. Although CAM in xerophytic plants allows for water conservation in dry environments, the pathway behind this metabolism (C4, Hatch/Slack/Korshak pathway) is fundamentally a carbon concentrating mechanism which increases the selectivity of rubisco by altering the CO_2:O_2 ratio within the chloroplast[5,18].

In 1981, diel acidity cycles similar to CAM were first observed in the aquatic lycophyte genus *Isoëtes*[6]. This metabolic process was named ‘aquatic CAM’ to highlight its similarity to xerophytic CAM acidity cycles. Here we instead will use the term ‘nocturnal carbon

accumulation' (NCA) to emphasize the differences between the behavior described in *Isoëtes* and that in xerophytic CAM plants. Further investigations of NCA in *Isoëtes* have demonstrated a high degree of plasticity. For example, *Isoëtes howellii* exposed to atmospheric CO_2 levels while growing terrestrially tend not to accumulate carbon nocturnally, while *I. karstenii* accumulates carbon regardless of environment, demonstrating that this behavior can be facultative or constitutive[7-9]. Since CAM serves as a carbon concentrating mechanism, CAM-like NCA in *Isoëtes* has been assumed to be a response to low carbon availability in aquatic ecosystems[2,6-9,12,18]. In eutrophic lakes hypocarbia is present only during the day because of diurnal photosynthesis and nocturnal respiration of aquatic algae and macrophytes; in oligotrophic lakes hypocarbia is generally continuous[10-12]. But, the theory that nocturnal carbon accumulation in aquatic plants is an adaptation to carbon limitation has hitherto remained largely hypothetical and correlative[6-12]. Furthermore, NCA in *Isoëtes* has been regarded as an adaptation by extant lineages to these selection pressures[7,18], rather than, as we suggest, an adaptation early in this ancient lineage, inherited by recent descendants.

To test the hypothesis that NCA is a direct response to carbon starvation, we grew terrestrial plants of two *Isoëtes* species in environmentally controlled growth chambers, starved plants of atmospheric CO_2 during the day and sampled plant pH from multiple plants for 24-hours. Diurnal hypocarbia induced diel acidity cycles in terrestrial *Isoëtes engelmannii* like those observed in xerophytic CAM plants and submerged aquatic *Isoëtes* (Fig. 4c). This provides direct evidence that CO_2 starvation can induce CAM-like NCA in *Isoëtes*, and supports the hypothesis that CO_2 limitation is a selective pressure on this behavior in extant *Isoëtes* species[7-11,13,18]. Moreover, the induction of NCA in terrestrial *Isoëtes* by atmospheric CO_2 starvation may support the hypothesis that CAM-like photosynthesis evolved early in terrestrial Isoetalean lineages in the Carboniferous in response to globally low atmospheric CO_2 levels, not more recently in response low aquatic CO_2 levels[2,7-11,13,18].

In our first series of field measurements from May to September, we measured morning and evening pH in *Isoëtes engelmannii* leaves, roots, and corms, monthly. (Fig. 1a; Fig. 2). We found that in the field, submerged plants of *I. engelmannii* accumulated carbon on a diel cycle (Fig. 2; Fig 3). The mean morning pH of the leaves throughout the summer was 3.71 and the mean evening pH was 4.90 (Fig. 2). Non-photosynthetic organs (roots and corms) did not accumulate carbon nocturnally (Fig. 3); corms: mean morning pH 5.94, mean evening pH 5.97; roots: mean morning pH 6.32, mean evening pH 6.36 (Fig. 3). Upon recession of the shoreline in July, many of the plants were left growing terrestrially, exposed to atmospheric conditions (Fig. 1). In the plants growing terrestrially, pH had higher variability (Fig. 1d; Fig 3), the difference between morning and evening pH was insignificant (Fig. 2). This supports prior observations that *I. engelmannii* accumulates carbon nocturnally only when submerged[7].

For lab manipulations, we collected *I. engelmannii* from the same population measured in the field as wells as specimens of *I. tuckermanii*, a related species that generally grows completely submerged. All plants collected were brought into the lab and cultivated in climate-controlled growth chambers. Under ambient CO_2 levels the diel pH variation of submerged individuals of *I. engelmannii* was similar to submerged individuals in the field (Fig. 4a); leaf mean morning pH was 4.58, and mean evening pH was 5.81. Submerged plants of *I. tuckermanii* had a less dramatic diel shift in pH, compared to *I. engelmannii* (Fig. 4a, 4d), mean morning pH

was 3.89, and mean evening pH was 4.70. When emergent, as expected from the field experiments, individuals of *I. engelmannii* no longer displayed a diel shift in pH; (Fig 4b; Fig. 2); mean morning pH was 5.31, mean evening pH was 5.46. *Isoëtes tuckermanii,* however, showed a more dramatic diel change in pH, when emergent (Fig. 4e); mean morning pH was 4.15, mean evening pH was 5.84. These results demonstrate different NCA behavior in these two species, as has been previously documented in *Isoëtes*[7]. *I. engelmannii* induces nocturnal carbon accumulation when submerged but not when emergent, while *I. tuckermanii* demonstrated constitutive NCA, with similar diel variation in pH irrespective of water depth, and only a slight increase in NCA when emergent compared to when submerged.

We next grew the plants with diurnal CO2 starvation and nocturnal enrichment to mimic the CO2 conditions in a eutrophic lake. *Isoëtes engelmannii* individuals grown under these conditions again showed a diel cycle in pH similar to that observed in the submerged specimens in lab and field (Fig. 2; 3a; 3c). For instance, in two independent experiments, we measured a mean morning pH of 4.69, mean evening pH was 6.37 (Exp. 8); mean morning pH 4.85, mean evening pH 6.18. The magnitude of pH change in the terrestrial plants grown under diurnal CO_2 starvation was similar to that in submerged plants in the field experiments, though slightly dampened (Fig. 3; Fig 4c). This may be due to the fact that predawn CO_2 concentrations in the field in eutrophic lakes and ponds can exceed 2500ppm[10], and the maximum CO_2 enrichment during our experiment approximated ambient atmospheric concentrations of 400 ppm. (Fig. 3c).

When grown under these manipulated CO_2 conditions, *I. tuckermanii* continued to show nocturnal carbon accumulation (Fig. 1c; Fig. 3f) with a mean morning pH of 4.12, and a mean evening pH of 5.98. The pH fluctuation of *I. tuckermanii* during diurnal hypocarbia did not differ from that of the terrestrially growing plants in ambient CO_2 (Fig. 3e), suggesting that this species is obligate in its NCA. Unlike *I. engelmannii*, the individuals of *I. tuckermanii* we examined had no stomata, a possible explanation of this constitutive behavior. These results provide a possible explanation for the plasticity of NCA in the genus *Isoëtes* observed in the past[7,12]. Moreover, we show that laboratory-based experimental modification of natural conditions can replicate field conditions and that carbon starvation imposed on terrestrial plants in the lab produces a diel change in pH comparable to the change observed in submerged aquatic plants.

Prior discussions of the adaptive significance of NCA in *Isoëtes* have focused on aquatic CO_2 limitation as a selective force[2,6-12,18]. Our experiments now show that terrestrial carbon limitation has the same effect. Low atmospheric CO_2 was a notable feature of the Carboniferous,[15-17] a time when Isoetalean lycopsids formed a substantial part of the terrestrial flora. In the Carboniferous atmosphere[14-17], a carbon concentrating mechanism that increased carbon gain and minimized the photorespirative loss of energy and carbon in a high oxygen/low CO_2 atmosphere would have been particularly advantageous. Given that we induced NCA in terrestrial *Isoëtes* (Fig 3c), and that this behavior is observed in many *Isoëtes* species[7,12,18], it seems likely that NCA evolved in the early Isoetalean lycopsids during the low atmospheric CO_2 conditions of the Carboniferous period. If so, NCA in extant aquatic *Isoëtes* would represent an exaptation of a much older metabolic behavior rather than a more recent adaptation to low aquatic CO_2 levels. Final determination of the evolutionary timing of NCA in *Isoëtes* requires more complete consideration of the temporal variation and selective pressures of local aquatic and global atmospheric CO_2 levels, as well as the distribution of this behavior in extant *Isoëtes*

species. Quantification of NCA at the species level across the genus *Isoëtes* will require further phylogenetic and physiological work[7,18,20]. Examining a broader range of *Isoëtes* species and using phylogenetic comparative methods may reveal varied appearances in NCA throughout the extant genus[7,18], just as CAM has been found to evolve many times in xerophytic plants[13,19].

Our results presented here do support the hypothesis that NCA in *Isoëtes* was an early evolutionary behavior, not a specialized or derived adaptation[7,18]. NCA appears throughout the vascular plant tree, including among ferns, lycophytes, and angiosperms and may have evolved as much as 350 million years before CAM appeared late in the Miocene[13]. Therefore, it seems reasonable to consider NCA to be the major photosynthetic pathway for carbon concentration of which CAM is a more recent offshoot representing an adaptation to dry environments. This view seems to better reflect the evolutionary sequence and significance of these behaviors.

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Methods

Field Experiments: The Edmund Niles Huyck Preserve (hereafter ENHP) is located in Rensselaerville, NY, 425–525m elevation. Plants of *Isoëtes engelmanii* were studied from May-September in a population on the north shore of Lake Myosotis[21], 42°31'26.8" N 74°9'7.2" W. Three to eight samples composed of 5-6 plants were collected five times during the summer of 2018 from May-September (Fig. 2). During May, June, and July samples were collected only in the morning at 0600 and in the evening at 1800; in August 3-8 samples were collected every three hours from 0600--1800 and in September collections were made every three hours for a full 24 hours (1800--1800). All plants were cleaned of algae and soil in DI water in the lab, which was located 1km from the study site.

In the lab, leaves, corms, and roots were separated using scalpels and forceps and organs were blotted dry. At each time point leaves from 5-6 individuals of *I. engelmanii* were randomized and separated into 3-8 distinct samples. Leaves from each of the 3-8 samples were macerated using plastic dowels in 1.7mL Eppendorf tubes and 0.5mL of DI H2O was added to each sample.

Samples were resuspended by mixing using a vortex mixer at maximum intensity for 10 seconds, followed by centrifugation at 10,000 rpm for 10 seconds. The supernatant was carefully extracted using a pipette, and placed on the Horiba LAQUAtwin pH-22 meter, for pH readings. The pH was measured twice from each sample to ensure accuracy of the reading. The pH meter was cleaned with deionized water and allowed to drt between each sample. The pH meter was recalibrated between each time point using pH 4 and 7 standardized buffers.

Isoëtes engelmannii specimens collected in the field throughout summer 2018 in Lake Myosotis were cultivated in growth chambers in the Weld Hill Research Building of the Arnold Arboretum of Harvard University. In addition, populations of *I. tuckermanii* already growing in the greenhouse (collected from the NW shore of Lake Mattawa, in Orange, MA, 42°34'11.9" N 72°19'34.1" W), were used in the growth chamber experiments. For 2 months, plants were grown fully submerged at ambient CO_2 levels (~400ppm), at 20˚C, with a 12-hour photoperiod, 15µmolm^{-2} photosynthetically active radiation. While submerged pH was measured on a diel cycle following the field protocol (see above). Containers were then drained of water, and the plants were allowed to acclimate for 1-3 days. While plants were terrestrial under ambient CO_2 levels pH was measured every 3-5 hours for 24 hours following the aforementioned protocol. Plants were then moved to a growth chamber set to a diel cycle of atmospheric CO_2: 100ppm of CO_2 during the 12-hour photoperiod (the minimum obtainable in the chamber) and 400ppm (equivalent to ambient atmospheric levels) during dark period. Temperature and light intensity were not changed from the ambient CO_2 conditions. Starting at 0700, plants were harvested every 3--5 hours for 24 hours. At each time point leaves from 2-4 individuals of were randomized and separated into 2-3 distinct samples and pH was measured following the field protocol.

Data and materials availability: all data can be found in the Supplemental Information.

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Figure 1: **a**: Lake Myosotis study site during average water levels for most of the year. **b**: Lake Myosotis in July when water levels drop. **c** exposed plants of *I. tuckermanii* in lab conditions. **d**: exposed plants of *I. engelmanii in situ*.

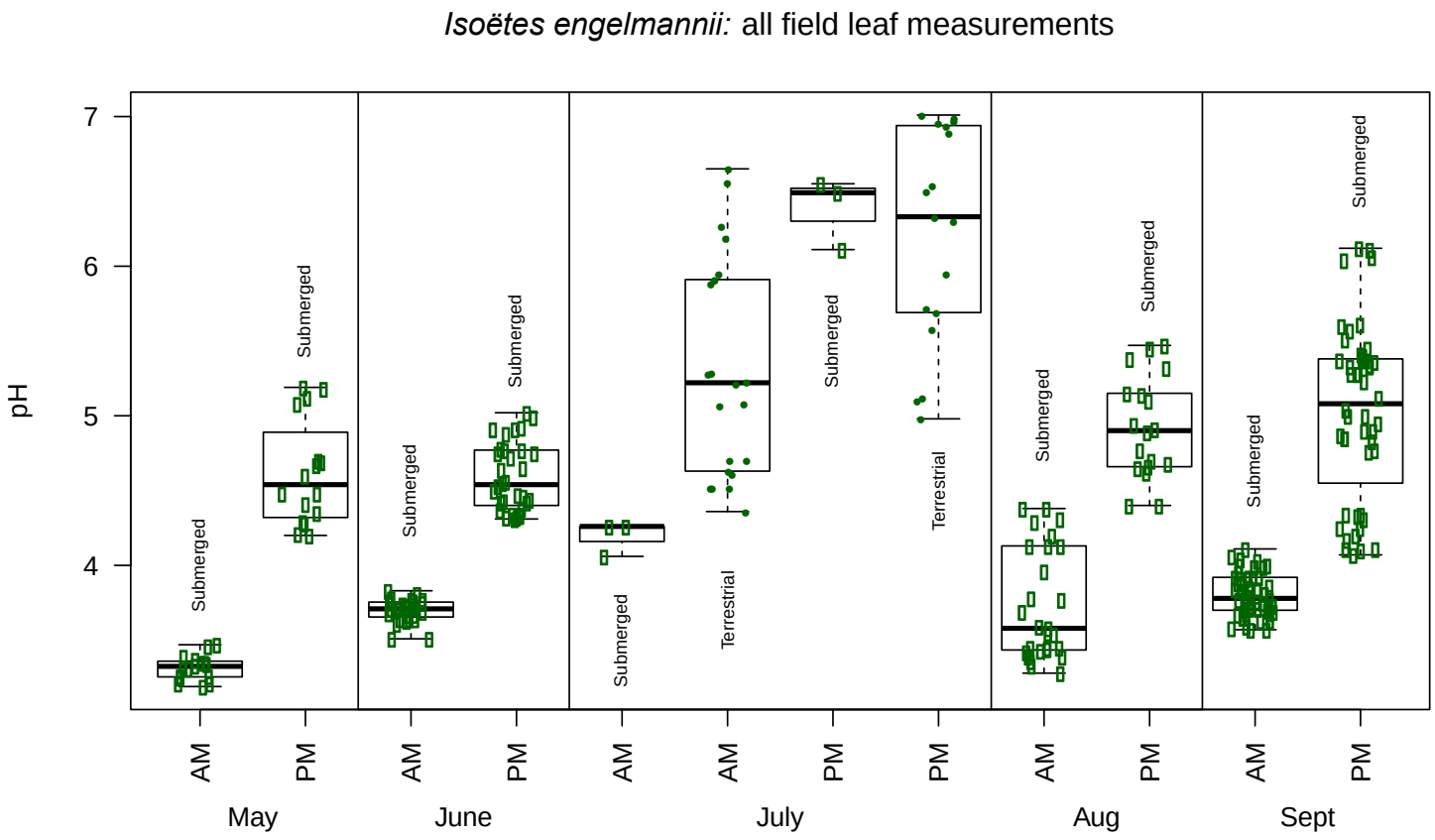

Figure 2: Field experiments. Diel pH measurements of leaves from field grown plants throughout the summer months. In July some plants were growing terrestrially and did not show significance differences in pH. Each point represents a single pH measurement of 3-8 samples comprised of pooled leaves from 5-6 individuals, pH was measured twice on each sample. During September more than two time points were measured (see Fig. 3).

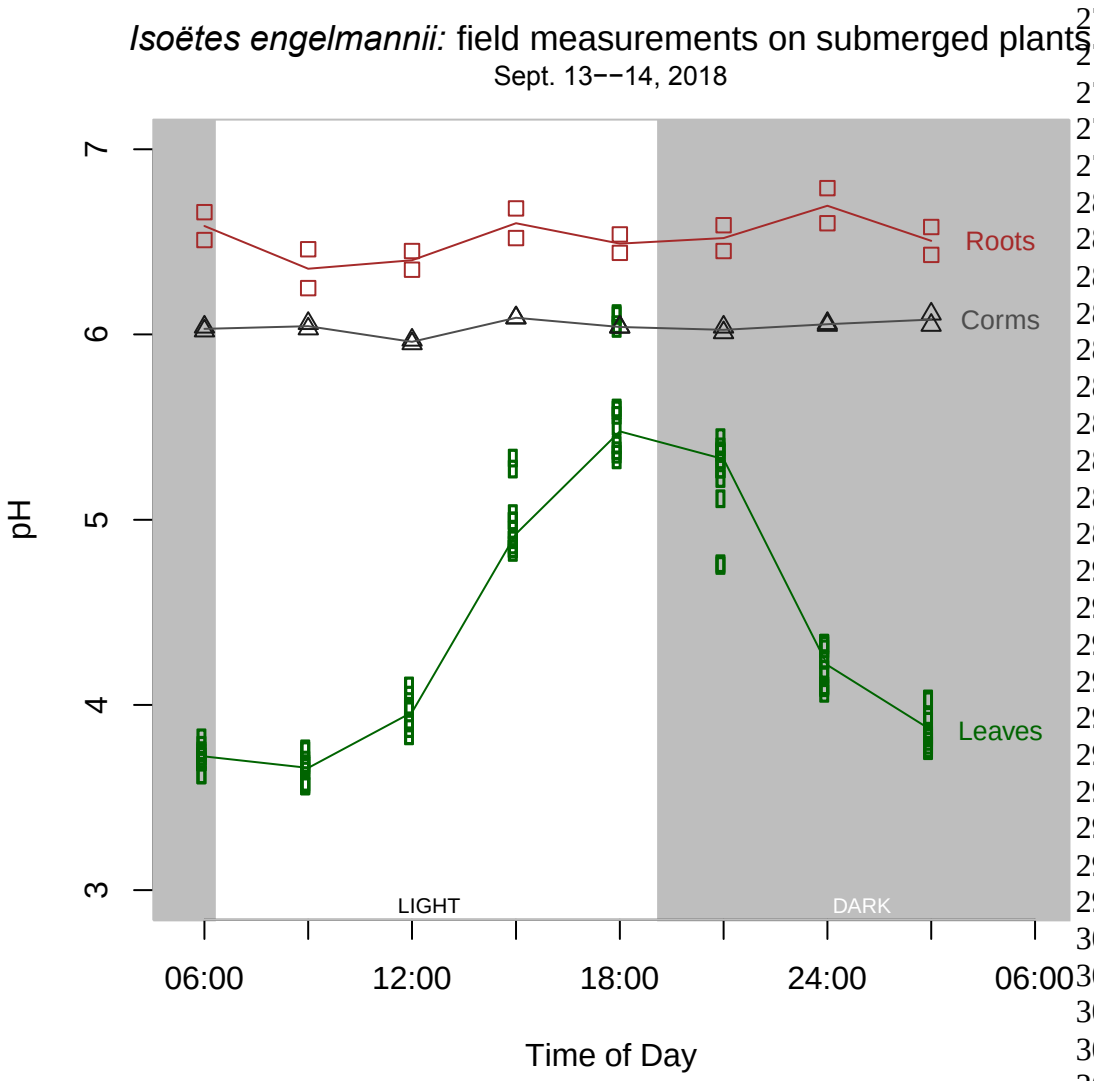

Figure 3: Field experiments. pH measurements of leaves, corms and roots, for 24-hours in September. A clear cycle in pH is demonstrated in leaves while corms and roots show no diel change. Each point represents a single pH measurement of 3-8 samples comprised of pooled leaves from 5-6 individuals, pH was measured twice on each sample.

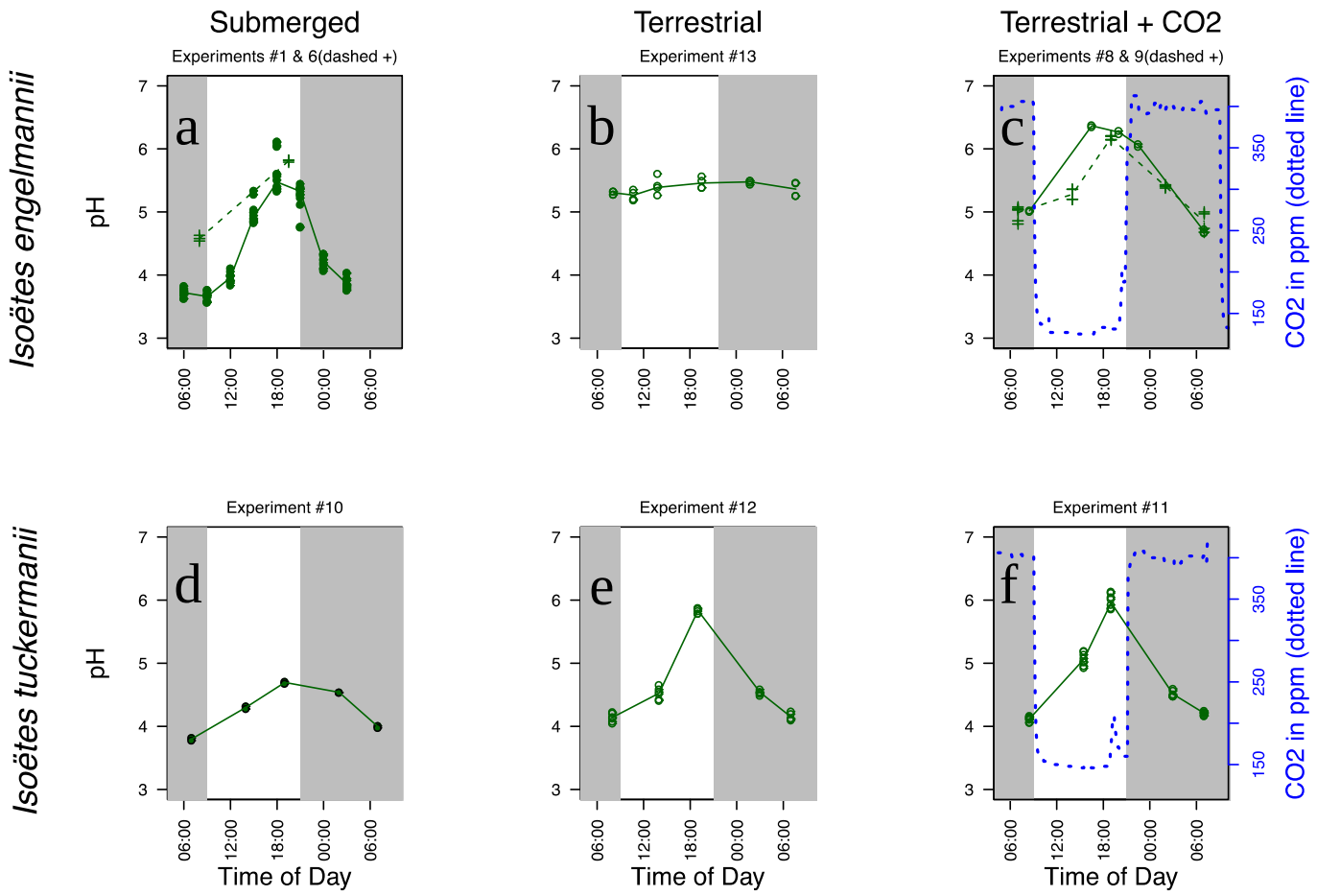

Figure 4: Laboratory experiments. Leaf pH measurements of *I. engelmanii* and *I. tuckermanii* under, **a, d**. submerged ambient CO_2 levels; **b, e**. terrestrial ambient atmospheric CO_2 levels; and **c, f**. terrestrial CO_2 manipulation. A clear diel change in pH was not observed in *I. engelmanii* when emergent but was induced upon CO_2 starvation. Solid circles represent submerged plants and open circles represent terrestrial plants. Each point represents a single pH measurement of 2-3 samples comprised of pooled leaves from 2-4 individuals, pH was measured twice on each sample.
*Circles in pane **a** are measurements from the field, crosses represent lab experiments. In pane **c** two separate 24-hour experiments were conducted (crosses and circles).