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Effects of 4 years of CO_2 enrichment on the abundance of leaf-galls and leaf-mines in mature oaks

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ABSTRACT

Since CO_2 enrichment alters the composition of live plant tissues, the ongoing global increase of atmospheric CO_2 concentration is expected to affect plant–animal interactions. Although this is a widely explored field, test systems studied so far have not included mature trees. Our study uses the Swiss Canopy Crane and the web-FACE CO_2 enrichment system in a 35 m tall temperate deciduous forest. We studied the *in situ* abundance of dominant leaf-galls and leaf-miners on 13 mature oaks before the treatment started, and in two sub-groups, one of which received CO_2 enrichment from 2001 to 2004. We expected that gall-inducing cynipids (*Neuroterus quercusbaccarum* and *N. numismalis*) would show similar loads with spangle-galls on the two tree groups, due to their capacity to manipulate their host plant and the quality of gall tissues. On the other hand, we expected that leaf-miners (*Tischeria ekebladella*) would show higher infestation on trees with high-quality (i.e. low C/N ratio, etc.) foliage leading to less mines on CO_2 enriched oaks. Partially in line with our hypothesis, spangle-gall density showed only a positive short-term pulse effect (*N. quercusbaccarum* only) and did not differ across treatments after 4 years. In contrast to our hypothesis, the leaf-miner species did not respond persistently to CO_2 enrichment either; it even showed higher infestation on CO_2 enriched oaks in the 1st year after the onset of enrichment. In conclusion, CO_2 enrichment had no lasting effect in all three taxa, despite the substantial and consistent change in leaf chemistry of oak due to growth in elevated CO_2.

1. Introduction

The ongoing global increase of atmospheric CO_2 concentration is expected to approximately double pre-industrial levels by the middle of the century (Houghton et al., 2001). This may have far-reaching consequences for interactions between insect herbivores and their host plants, since CO_2 enrichment often alters the composition of live plant tissues (Bezemer and Jones, 1998; Körner, 2000). Generally, elevated CO_2 levels cause higher C/N ratios, largely because of increased starch concentration and adjustments of photosynthetic machinery (Körner and Miglietta, 1994; Wilsey, 1996; Cotrufo et al., 1998) and/or

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increased growth, often resulting in a nitrogen dilution (Lincoln et al., 1993). Lowered plant nitrogen can have detrimental effects on insect herbivores, because it is generally regarded as a crucial limiting nutrient for phytophagous arthropods (Mattson, 1980; Strong et al., 1984), and because it can increase the production of carbon-based secondary chemicals such as phenolics (Agrell et al., 2000; Hartley et al., 2000), deterring herbivore feeding.

Despite the general wisdom that 'low plant N negatively affects insect herbivores' – for example, by increasing development time, by increasing consumption, or by decreasing pupal mass – existing studies indicate that there might be winners and losers among the large diversity of phytophagous insects in terms of species, as well as modes of mobility and feeding (Coviella and Trumble, 1999). However, compared to studies with chewing insects – i.e. those that have to cope directly with low plant tissue quality – other feeding-guilds have received less attention. These include sap-feeding (Docherty et al., 1997; Holopainen, 2002), cell-content feeding (Joutei et al., 2000), gall-forming (Stiling et al., 2002, 2003) and leaf-mining arthropods (Stiling et al., 1999; Johns and Hughes, 2002; Rossi et al., 2004).

The prediction of herbivore performance and herbivore damage in a future high-CO_2 world is further limited by the fact that the responses of species from various feeding-guilds to enhanced CO_2 concentration, have been mainly studied on herbaceous plants (Bezemer and Jones, 1998) and tree-seedlings (Docherty et al., 1997; Kopper and Lindroth, 2003a). Only a few studies are available from more natural communities (e.g. resprouting scrub-oak vegetation, Stiling et al., 1999, 2002, 2003) or intact forest understorey (Hamilton et al., 2004). In contrast to the common view that herbivore damage will increase under elevated CO_2 as a consequence of compensatory feeding due to lower foliage quality, these studies suggest that damage to trees may actually decrease. A mechanism for explaining this observation conclusively is not yet available (Knepp et al., 2005), but reduced food quality seems like a plausible reason. Tall mature trees in a natural forest have not yet been studied although closed tree stands behave differently than young expanding systems with regard to plant CO_2 responses (Körner, 2006). Given that forests cover more than 44 million km^2 (Forest Resources Assessment Programme, 2001) – a considerable part of the earth's terrestrial surface – and represent close to 90% of the terrestrial global biomass carbon pool (Körner, 2003), this means an enormous gap in our understanding of insect-plant relationships and their ecological consequences in a future high-CO_2 world.

The aim of our study was to monitor medium-term effects of CO_2 enrichment on the load of sessile herbivores in the canopy of mature temperate forest trees. Hence, we determined the abundance of dominant leaf-galls (spangle-galls induced by the cynipid wasps *Neuroterus quercusbaccarum* and *N. numismalis*) and leaf-mines (caused by the larvae of the moth *Tischeria ekebladella*) on freely colonized large oaks in a mixed forest in Switzerland, which received CO_2 enrichment from 2001 to 2004. We used the Swiss Canopy Crane (SCC) and a new CO_2 enrichment technique (web-FACE) applicable to tall forest trees. As opposed to free-living herbivores which can choose optimal foliage for development, the density of galls and mines is set at an early development stage depending on: (1) the oviposition-site selection by their mothers; and (2) the hatching success and performance of the eggs and larvae. While gall-forming insects can regulate the nutritive value of the plant tissues on which they feed to their own benefit (Stone et al., 2002), leaf-miners depend on leaf quality in the same way as do leaf-chewing insects and often respond with compensatory feeding to low quality food as induced by CO_2 enrichment (Cornelissen et al., 2003; Stiling et al., 2003). For specialist feeders, correct decisions during host selection behavior are of utmost importance and are guided by specific blends of volatiles (Dicke, 2000). Assuming that mothers would recognize optimum tissue quality for the development of their offspring, we expected gall-formers and leaf-miners to show differences in response to CO_2 enrichment. The former should not show any preference for high-CO_2-exposed or ambient-CO_2 exposed oaks, leading to similar loads with spangle-galls (due to their capacity to manipulate gall tissue quality). However, miners should show a distinct preference for trees under ambient CO_2 conditions leading to less mines on CO_2 enriched oaks. It is worth noting that gall-formation by cynipids on oak was found to deviate from responses in other insects (responded less) when tissue nitrogen concentration varied (Hartley and Lawton, 1992). These authors demonstrated in a nitrogen fertilizer experiment that *N. quercusbaccarum* actively controlled nitrogen concentration of the spangle-gall tissue, regardless of whether the overall leaf tissue had high or low N concentrations. The oak trees tested here responded with a significant increase of the C/N ratio and carbohydrate concentration when exposed to elevated CO_2 (Körner et al., 2005), combined with a host of other leaf quality changes, offering ideal, *in situ* test conditions for the above hypothesis.

2. Materials and methods

2.1. Study area and CO_2 enrichment

We used the Swiss Canopy Crane, located in a highly diverse mixed forest stand, approximately 15 km south of Basel, Switzerland (47°28′N, 7°30′E) at an elevation of 550 m a.s.l. (Körner and Zotz, 2003). The climate is characterized by mild winters and moderately warm summers with a growing season of approximately 165 days (end of April to early October). Mean annual temperature is 10 °C (January 2.1 °C, July 19.1 °C), and total annual precipitation averages 990 mm. Soils are of the rendzina type on calcareous bedrock with a profile depth of 30 cm; pH is 5.8 in the top 10 cm. The forest is 80–120 years old with a canopy height of 32–38 m, consisting of seven deciduous (*Fagus sylvatica*, *Quercus petraea*, *Q. robur*, *Tilia platyphyllos*, *Carpinus betulus*, *Acer campestre* and *Prunus avium*) and four coniferous species (*Larix decidua*, *Picea abies*, *Pinus sylvestris* and *Abies alba*) in reach of the crane. The SCC was erected in March 1999 by lowering it from a helicopter into a natural gap in the forest. Thus disturbance to the site (soil compaction, tree-felling, etc.) was avoided. The crane has a jib of 30 m and permits sampling of an area comprising 2800 m^2 with 64 tall trees.

Canopy CO_2 enrichment was performed with a new free-air CO_2 enrichment system for tall trees (web-FACE, Pepin and Körner, 2002). Pure CO_2 gas was released from ca. 8.5 km of 4 mm tubes woven into the upper canopy (>20 m) of a group of 14 trees (between 300 and 1000 m of tubing per tree depending on crown size). Emission occurred during daylight hours throughout the growing season through small laser-punched holes (0.5 mm diameter) spaced at intervals of 30 cm. CO_2 concentration within the experimental areas was computer-controlled in connection with a 24 channel infrared gas analyser (IRGA). Seasonal daytime mean CO_2 concentration was 540 μmol mol^{-1} CO_2 in the treated area, and 375 μmol mol^{-1} in the control area.

CO_2 enrichment distinctly affected the chemistry of oak leaves at the SCC site. Water content, specific leaf area, fiber and protein concentration decreased significantly. Sugars, starch (non-structural carbohydrates) and condensed tannins showed higher values on CO_2-exposed trees. Nitrogen concentration (% dry weight), however, did not respond significantly to CO_2 treatment (Hättenschwiler and Schafellner, 2004; Körner et al., 2005). With the exception of the nearly unaffected nitrogen concentration, the common expectation, that herbivore arthropods would encounter food of generally lower quality on high-CO_2 exposed trees was clearly met in the experiment.

2.2. *Sampling and study organisms*

Before the CO_2 treatment (July 2000) and after CO_2 enrichment commenced in September 2000 (July 2001, July 2002, July 2004) we took a census of 13 oaks of which three large oaks were in the CO_2 enriched zone. In each tree we chose a random position in the upper (20–30 m) and top part (>30 m) of the canopy. On these we counted and identified galls and mines on the 50 most terminal leaves of three randomly chosen branches, yielding a total of 3000 checked leaves per year (foliage was lacking in some trees in some layers).

The dominant gall-formers were the cynipid wasp species *N. quercusbaccarum* and *N. numismalis*; other leaf-galls appeared in negligible numbers. These species attack only oaks; they are heterogonous and the eggs laid by their sexual generation in late spring/early summer induce so-called ‘‘spangle-galls’’ on the leaves. They contain a larval chamber and nutritive zone, protected by a layer of sclerenchyma and surrounded by thin-walled parenchyma cells. Spangle-galls drop to the ground in autumn, and the agamic generation hatches from them in early spring (Askew, 1984; Stone et al., 2002). The only dominant leaf-miner was *Tischeria ekebladella*, a moth from the family Tischeriidae, which forms irregularly-shaped mines under the upper epidermis of the leaves of oaks and chestnut. Like the cynipids, their larvae overwinter and hatch in spring (Jordan, 1995).

In addition to available general phytochemistry data of enriched and not-enriched oaks, we analysed the concentration of carbon and nitrogen of leaves with spangle-galls of *N. quercusbaccarum* in 2000 and 2001, as well as of the spangle-galls themselves. Approximately 800 galls were collected each year in September in the top canopy; the galls and the leaves bearing them (approximately 300) were dried at 105 °C for 24 h, ground separately in a ball mill and homogenized, yielding two homogenates (galls, leaves) per tree per year. We determined C and N concentration (expressed as % of dry weight) of 2–5 mg of the homogenates in triplicate with a Carlo-Erba C/N Analyser 1500 N using atropine as a standard.

2.3. *Statistical analysis*

Since susceptibility of oaks to attack by cynipids and leaf-miners varies individually among trees (Kampichler and Teschner, 2002; Cornelissen and Stiling, 2008) and is highly consistent across trees between years (Askew, 1962; Rosenthal and Koehler, 1971; Hartley and Lawton, 1992), trees were considered the experimental unit. Statistical analysis based directly on the abundance data of the galls and leaf-mines is hampered by the fact that individual levels of susceptibility would override any CO_2 effect. Thus, for each tree we determined its arcsin-transformed proportion of galls and leaf-mines of each species of each year. Differences between enriched and control trees were tested by a linear mixed effects model for repeated measures data estimated by restricted maximum likelihood (Everitt and Hothorn, 2006) using the function lme (Venables and Ripley, 1999) of the statistical programming environment R (R Development Core Team, 2006) with CO_2 and year as fixed effects, trees as random effect and infestation in 2000 as covariate. Due to several strong storms, some layers in some trees were damaged during the investigation period. In order to keep a balanced design we included only those trees in the analysis that were not damaged. Hence, we analyzed nine trees under ambient CO_2 conditions and three trees under enriched CO_2 conditions in the upper canopy.

The linear mixed effect model was also used to test differences in gall, leaf carbon, nitrogen concentration, and resulting C/N ratios between the enriched and control trees, with CO_2 as fixed effect, trees as random effect and C, N and C/N values, respectively, in 2000 as covariate. Due to small sample sizes, and in order to reduce the probability of a type II error, we assumed differences to be significant at $p < 0.1$.

2.4. *Limitations and potentialities of the study design*

Doing large-scale CO_2 research on tall trees and their herbivores bears several limitations: (i) CO_2 treatment cannot be randomly assigned among single tree individuals within the reach of a canopy crane; the mechanical forces of storms and branch movements avoid any attempt of dividing a stretch of forest with screens or the like. Thus, the only possibility is to establish a CO_2 enriched zone and an ambient CO_2 zone. (ii) Statistical power is low due to low sample size (=number of trees within reach of the crane) of these otherwise very large trees, and the probability to detect statistically significant effects is limited. On the other hand, research at this scale is the only possibility to gather any information on the responses of mature forest ecosystems – or with the words of Oksanen (2001): ‘‘appropriate scale must always have priority over replication’’. In our case we profited from the possibility to study our test trees before the treatment began, thus, our analysis accounts for any pre-treatment difference, to as far an extent as such experimental conditions permit.

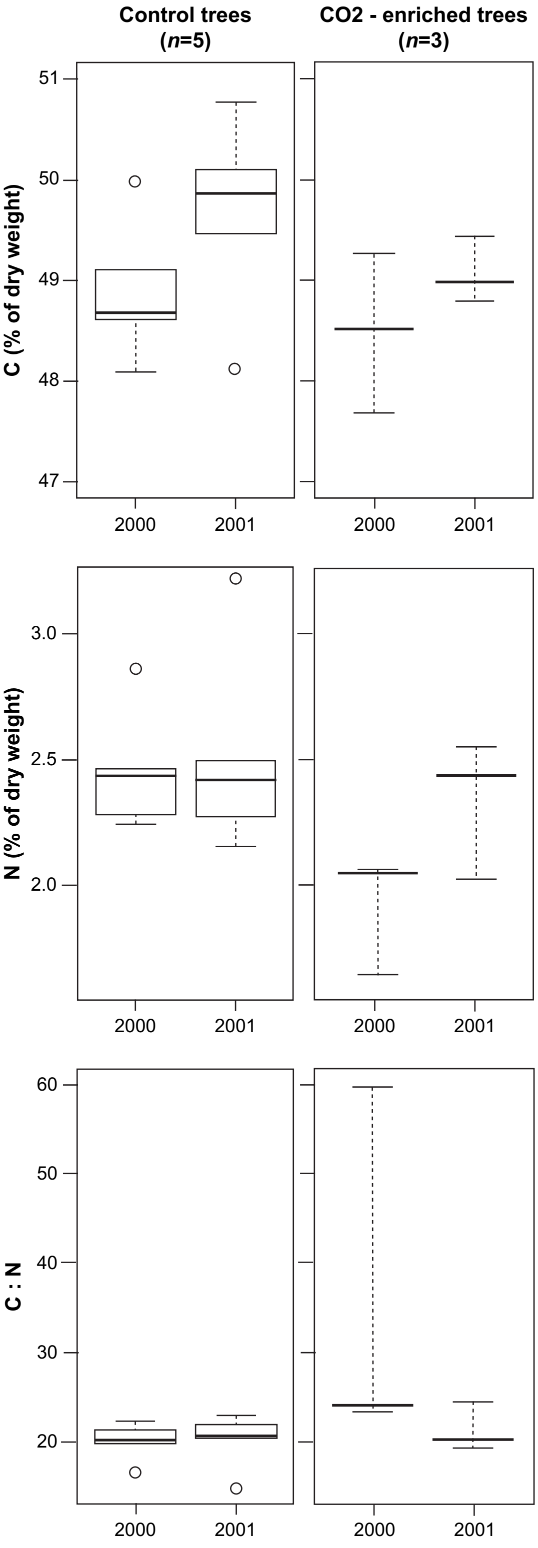

Fig. 1 – Carbon and nitrogen concentration and C:N ratio of leaves bearing *N. quercusbaccarum* spangle-galls on control trees and CO_2 enriched trees at the Swiss Canopy Crane crane site. Boxplots show medians, quartiles, range and outliers (data points with a distance from the rest of data of at least 1.5 times the interquartile range). Quartiles were omitted when *n* (number of trees in the analysis) was <5.

Table 1 – Effect of the CO_2 treatment of trees at the Swiss Canopy Crane site on the concentration of C and N and on the C/N ratio of leaves bearing spangle-galls as well as on infestation by *N. cuercusbaccarum*, *N. numismalis* and *T. ekebladella* calculated by mixed model ANOVAs for repeated measures data. df, degrees of freedom; *t*, value of the *t*-statistic; *p*, error probability. Effects were supposed to be statistically significant at $p < 0.1$

	df	*t*	*p*
Leaf C concentration	5	0.726	0.500
Leaf N concentration	5	−2.675	0.044
Leaf C:N ratio	5	0.595	0.578
N. quercusbaccarum	9	−1.955	0.082
N. numismalis	9	−0.574	0.580
T. ekebladella	9	−2.713	0.024

3. Results

3.1. *Leaf and gall phytochemistry*

Carbon concentration, nitrogen concentration and the C/N ratio of leaves bearing spangle-galls of *N. quercusbaccarum* showed considerable interannual variation between 2000 and 2001 (Fig. 1). While interannual carbon variation was independent of CO_2 treatment, CO_2 had a significant effect on nitrogen concentration (Table 1): nitrogen concentration of leaves from control trees maintained a constant value, but increased in leaves under enriched CO_2 conditions relative to its value prior to the onset of CO_2 enrichment. C/N ratio was not significantly affected by CO_2 treatment (Table 1, Fig. 1).

N. quercusbaccarum gall carbon and nitrogen concentration and the C/N ratio also varied between years (Fig. 2). However, due to small sample size (some trees did not bear galls in 2001), we refrained from performing a statistical analysis. A visual inspection of the data, however, did not yield any indications that gall phytochemistry might have been affected by CO_2 enrichment.

3.2. *Gall and mine infestation*

Numbers of leaf-galls induced by *N. quercusbaccarum* and *N. numismalis* and leaf-mines of *T. ekebladella* were highly variable between years (Table 2). CO_2 exerted significant effects on infestation by *N. quercusbaccarum* as well as of *T. ekebladella* (Fig. 3, Table 1). This effect, however, was not a persistent one and was found only in the 1st year of CO_2 enrichment (Fig. 3). There was no effect at all on infestation by *N. numismalis*.

4. Discussion

The response of cynipid spangle-galls confirmed our initial hypothesis ("no effect on cynipid infestation") only partially: while our analysis was designed to control for pre-treatment differences between enhanced CO_2 trees and ambient CO_2 trees, we still did not find persistent differences in oviposition preferences and/or eggs and early instar survival – the mechanisms that determine infestation – between oaks grown

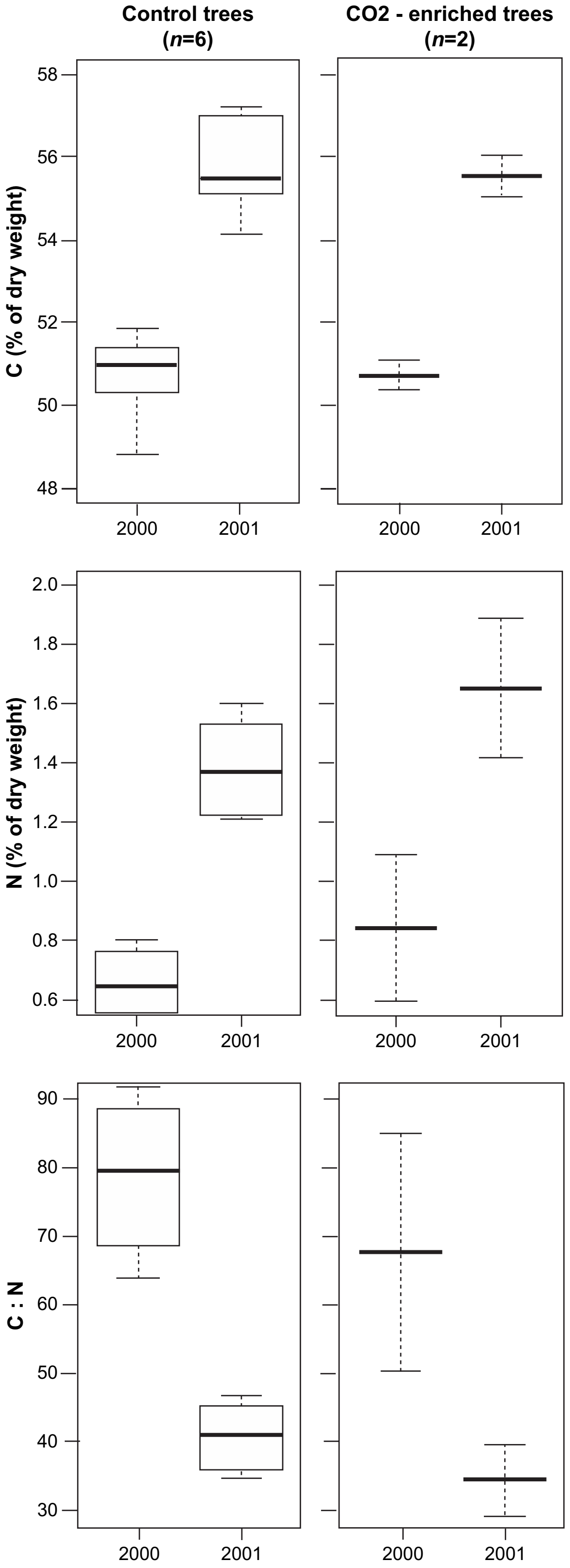

Fig. 2 – Carbon and nitrogen concentration and C:N ratio of spangle-galls of *N. quercusbaccarum* on control trees and CO_2 enriched trees at the Swiss Canopy Crane crane site. Boxplots show medians, quartiles, range and outliers (data points with a distance from the rest of data of at least 1.5 times the interquartile range). Quartiles were omitted when *n* (number of trees in the analysis) was <5.

Table 2 – Abundance of spangle-galls and leaf-mines collected on 13 oaks at the SCC site near Basel expressed as number per 100 leaves. Only leaves from canopy layers that were not damaged by storms during the study period (5 years) were included (see Section 2 for detailed description) resulting in a annual sample size of 2950 leaves

Year	*N. quercusbaccarum*	*N. numismalis*	*T. ekebladella*
2000	30.07	9.36	0.95
2001	18.54	4.31	0.61
2002	6.47	20.61	0.00
2004	13.59	72.71	3.02

under different atmospheric CO_2 concentration; we observed, however, a significant positive short-term pulse effect of CO_2 enrichment on *N. quercusbaccarum* infestations. Our data also show that as expected, larvae of *N. quercusbaccarum* manipulated host tissue quality: as opposed to the general trend in the CO_2 enriched area of the SCC site and in contrast to most studies that demonstrated a decrease of N with increasing CO_2, leaves bearing galls showed higher nitrogen concentration on high-CO_2 trees (considering N concentration prior to the beginning of CO_2 enrichment as a covariate). This also contrasts with the findings of Hartley and Lawton (1992), who reported higher nitrogen concentration (% dry weight) on ungalled leaves compared with galled ones. However, there were no indications for differences in N concentration and C/N ratios between galls on oaks in enriched and ambient CO_2. Nitrogen concentrations were within the range reported by Hartley and Lawton (1992) for *N. quercusbaccarum* and for galls by another cynipid wasp, *Andricus lignicola*. Despite their potential of host manipulation, carbon and nitrogen concentration and C/N ratios of the galls are not necessarily constant over time; their distinct interannual variability must be due to other mechanisms than CO_2 enrichment which were not investigated in our study (e.g. different soil nitrogen availability, 2003 drought). These mechanisms seem to easily override any atmospheric CO_2 concentration effect. Due to the smaller abundance of spangle-galls of *N. numismalis* we had no data available on their chemistry: however, we also assume host manipulation to be responsible for the lack of infestation differences between high-CO_2 and ambient-CO_2 oaks.

It is more difficult to interpret the initial positive response of *N. quercusbaccarum* to CO_2 enrichment. CO_2 is an attractant for some herbivorous insects and helps determine host location (Stange, 1997; Bernklau and Bjostad, 1998). Females of cynipid species seem to be more mobile than assumed earlier (Kampichler and Teschner, 2002) and could have actively moved to tree crowns within the CO_2 enriched area. We doubt, however, that the higher infestation with galls of *N. quercusbaccarum* and mines of *T. ekebladella* on CO_2 enriched trees can be simply explained by the same effect: (1) cynipid wasps are well known for their highly selective host choices, showing distinct preferences for certain trees within a stand (Askew, 1984; Stone et al., 2002); cynipid wasps have even been labeled ‘‘nature’s most sophisticated herbivores’’ (Shorthouse et al., 2005). (2) Higher infestation in 2001 may have been caused by females showing a preference for oviposition on CO_2 enriched trees due to some change in infochemical

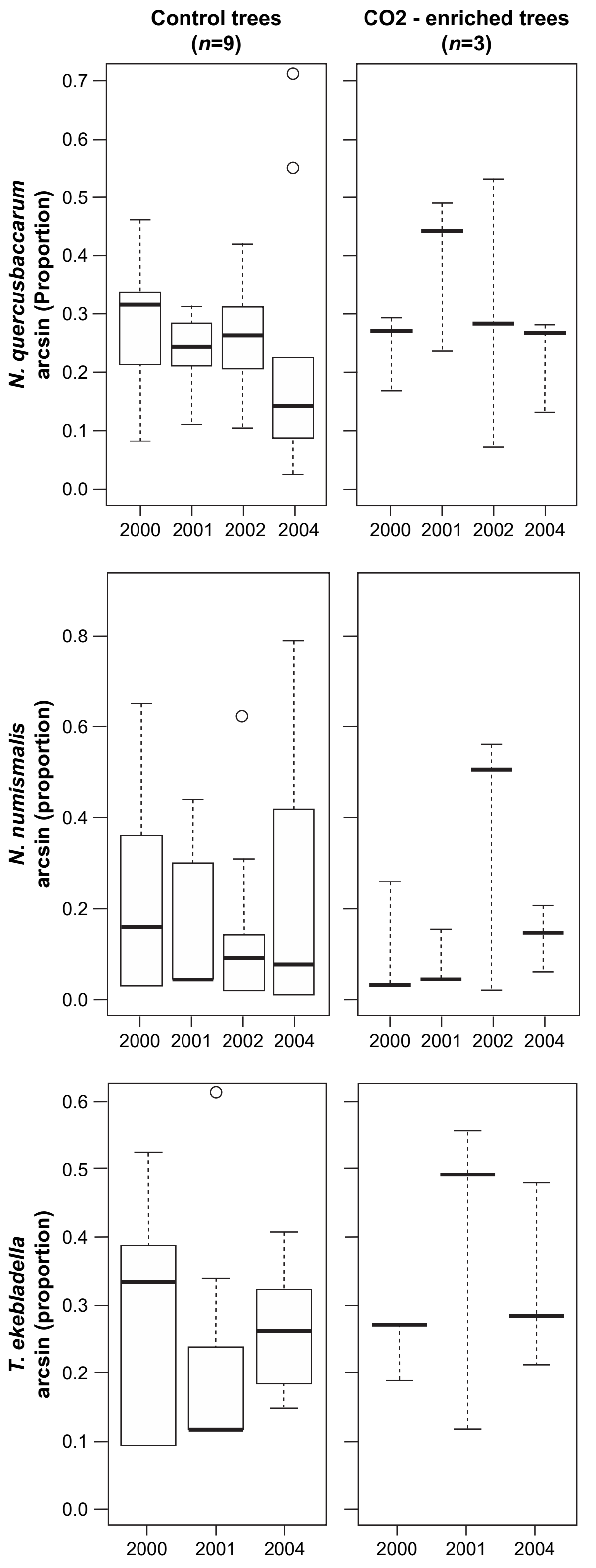

Fig. 3 – Infestation by *N. quercusbaccarum*, *N. numismalis* and *T. ekebladella* of control trees and CO_2 enriched trees at the Swiss Canopy Crane site. Boxplots show medians, quartiles, range and outliers (data points with a distance from the rest of data of at least 1.5 times the interquartile range). Quartiles were omitted when *n* (number of trees in the analysis) was < 5.

composition or gall-induction. Early instar survival could have been higher on CO_2 enriched trees due to better foliage quality (leaf nitrogen had increased, Fig. 1). Hartley and Lawton (1992), however, have shown that the performance of *N. quercusbaccarum* is not improved by enhanced nitrogen levels; therefore we doubt the "better foliage quality" hypothesis.

T. ekebladella leaf-miners were not persistently affected by elevated CO_2, in contrast to our prediction ("less mines on CO_2 enriched oaks"). Moreover, the observed transitory effect did not cause higher infestation on oaks grown under ambient conditions but on oaks exposed to CO_2 enrichment, that is, higher infestation on lower quality leaves. The majority of studies on leaf-miners on woody species under elevated CO_2 conditions showed compensatory feeding and declining abundance, for example, (Stiling et al., 1999, 2002, 2003) and Cornelissen et al. (2003) on oak, and Kopper and Lindroth (2003b) on aspen (see also Johns et al., 2003, on *Lantana camara*, a tropical perennial woody shrub). Some authors have also reported increased host plant induced mortality of herbivores, as well as higher attack rates by natural enemies (Stiling et al., 1999, 2003). The latter is probably due to longer duration of development and longer exposure to searching predators or parasitoids. Higher per capita feeding rates and longer development are also well-known patterns for leaf-chewing arthropod herbivores (Bezemer and Jones, 1998), which reflect the similarity of effects of CO_2 induced low quality plant tissue on leaf-chewers and leaf-miners. We studied large-scale canopy patterns over several years and did not include the investigation of leaf-miner performance. However, we can safely assume that the above-mentioned effects on arthropod herbivores also are valid at the SCC site. If this is so, then why do the *T. ekebladella* females fail to recognize optimum hosts although fecundity and other measures of insect performance heavily depend on host plant quality (Awmack and Leather, 2002)?

A look at published studies shows that leaf-miners do not seem to be very reliable in their host choice. Kopper and Lindroth (2003b) demonstrated decreased colonization rates under elevated CO_2 conditions although larval performance did not differ between host plants under different CO_2 levels. In contrast, Johns and Hughes (2002) found no discrimination between foliage grown at different CO_2 levels although larval performance was negatively affected by elevated CO_2. Obviously, these observations contradict the assumption made above that mothers should be able to recognize optimum oviposition sites. Awmack and Leather (2002) describe the various strategies available to female insects encountering poor-quality hosts (reduction of number of eggs laid, adjusting size or nutritional concentration of the eggs, etc.) but notice that although some species use host plant quality to make decisions about number and quality of offspring deposited, many species do not. Therefore, many insects – and among them leaf-miners – make oviposition choices that appear erroneous (Courtney and Kibota, 1990; Faeth and Hammon, 1996) and fall into ecological traps (Gilroy and Sutherland, 2007).

Yet, undisturbed and mature natural tree communities might respond in a manner different from plants developing under controlled experimental conditions and may offer surprises (Knepp et al., 2005). In contrast to results for high-CO_2 exposed tree seedlings (Cotrufo et al., 1998; Norby et al., 1999), the significant increase in non-structural carbohydrates in oak foliage did not cause a significant reduction in leaf nitrogen concentration at the SCC site (Hättenschwiler and Schafellner, 2004; Körner et al., 2005). Other species, such as gypsy moth, that feed in the canopy of the SCC site, showed 30% reduced larval growth rates when feeding *in situ* on high-CO_2 exposed oak, but they grew 29% faster on high-CO_2 exposed hornbeam (*Carpinus betulus*; Hättenschwiler and Schafellner, 2004).

5. Conclusion

Although elevated CO_2 reduced foliar quality of mature oaks in terms of various parameters (water content, proteins, non-structural carbohydrates, tannins, etc.) at the SCC site (Körner et al., 2005), on the long term, their load with cynipid spangle-galls and leaf-mines of *T. ekebladella* was not distinguishable from that in oaks exposed to ambient CO_2 after 4 years of treatment. On the short-term, we observed even a positive response to CO_2 enrichment. This is most likely because the gall tissue composition is highly controlled for constant quality. It came as a surprise that leaf-miners also did not respond, – or if so, they even showed a preference for high-CO_2 oaks –, in contrast to previous tests with young plants, perhaps having to do with effects of age and size of host trees. The positive transitory response of *N. quercusbaccarum* and *T. ekebladella* to CO_2 enrichment at the beginning of the experiment highlights the importance of long-term observations of such plant–herbivore interactions in elevated CO_2.

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