Carbon starvation by light deprivation does not constrain the ability of young pines to produce induced chemical defences in response to a bark-chewing herbivore

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Appendices

Appendix A. Summary of the mixed models

Appendix B. Temporal dynamics of weevil feeding rate

Appendix C. Effect of light deprivation on the concentration of chemical defences in undamaged plants

Appendix D. Within plant allocation of chemical defences

Appendix E. Effect of light deprivation on the within-plant variation in the concentration of chemical defences.

.Appendix F. Multimedia file with a quick video summary of the paper.

Highlights

- Young pines of three [species](#) were exposed to insect herbivory in darkness during 7 d

- In all species, pine weevil fed more in darkness than under natural sunlight

- Young pines increased their chemical defences proportionally to the weevil feeding

- Light-deprivation did not constrain the ability of pines to produce induced defences

- Current assimilated C does not appear to be mandatory for synthesis of resin induced defences

Abstract

Pine tree chemical defence is based on constitutive and inducible carbon-rich substances (mainly oleoresin and phenolic compounds) present in pine tissues in conspicuously high concentrations. Resistance at early stages is essential for future fitness, and allocation of carbon resources to chemical defences may signify a relevant carbon sink for juvenile pines. However there is still poor knowledge about the extent to which allocation priorities of recently assimilated and stored C pools to chemical defences are plastic in response to biotic stress. We aimed to determine the extent to which the production of induced chemical defences in pine trees in response to herbivory depends on the availability of current photosynthates or if they can be built upon stored C pools. Two years-old trees of three pine species (*P. pinaster*, *P. sylvestris* and *P. radiata*) were exposed to real herbivory by the pine weevil *Hylobius abietis*, a bark-chewing insect, during one week under conditions of either natural sunlight or complete light deprivation. Weevil damage and the concentration of chemical defences in the whole stem were measured in a time-course design with destructive sampling. We analysed, using an ANCOVA approach, whether the ability to increase the concentration of chemical defences in response to weevil damage was altered by light deprivation. We hypothesised that if induced defences are built mainly upon current photoassimilates, then carbon starvation by light deprivation should strongly hamper the production of new defences. For all pine species, concentration of non-volatile resin significantly increased in response to weevil damage, which was more intense under dark conditions. ANCOVA analysis showed that light deprivation did not constrain the ability of young

53 pines to respond to weevil damage by increasing non-volatile resin content in the stems.
54 No significant covariation was found for total polyphenolics. By evaluating chemical
55 defences in the whole stem, and restricting C assimilation by complete light deprivation,
56 we can infer that young pines were able [to use other carbon sources than current](#)
57 [photosynthates, likely stored carbon pools, for *de novo*](#) synthesis of induced chemical
58 defences in response to insect herbivory. [Disentangling](#) the plasticity in the use of C
59 sources for the synthesis of induced defences is crucial for understanding the ability of
60 pine trees to [respond](#) to multiple stressors in a changing environment. [Please refer to the](#)
61 [video summary \(Appendix F\) for a quick overview.](#)

63 **Keywords**

64 Carbon allocation, carbon partitioning, *Hylobius abietis*, induced defences, light
65 deprivation, pine weevil, *Pinus spp.*

67 **1. Introduction**

68 Pine chemical defence is founded in the production and accumulation of a mixture of
69 carbon-rich compounds, mainly terpenes and phenolics, that serve as wound-sealant,
70 antifeedants or deterrents, providing resistance against insect herbivores (Eyles et al.,
71 2010; Franceschi et al., 2005; Moreira et al., 2014). Pests and diseases can cause severe
72 damage and extremely high mortality rates at the juvenile stage (Nordlander et al.,
73 2011; Zas et al., 2011), and therefore defence against biotic threats at early stages is
74 essential for pine fitness (Wainhouse et al., 2005; Zas et al., 2011). Terpenoid and
75 phenolic compounds are present in all pine tissues in conspicuously high
76 concentrations, in the order of 10-100 mg of secondary chemicals per gram of d.w.
77 tissues (Moreira et al., 2014; Sampedro et al., 2011a). The production of both types of

defences therefore signifies a relevant carbon sink for juvenile pines (Eyles et al., 2010; Gershenzon, 1994).

Pines have both constitutive defences, which are present in absence of attack, and induced defences, which are triggered after biotic challenge (Franceschi et al., 2005; Mumm and Hilker, 2006). In pines, herbivore-induced responses involve a rapid increase in the activity of terpene synthases (e.g. Funk et al., 1994; Lewinsohn et al., 1991; Steele et al., 1995) and in the concentration of major chemical defences (Zas et al., 2014; Zhao et al., 2011), qualitative changes (Moreira et al., 2013; Zhao et al., 2011) and even anatomical modifications such as the genesis of traumatic resin ducts (Franceschi et al., 2005; Moreira et al., 2015). An increasing number of studies have shown the critical relevance of those induced defences for effective protection against particular insect herbivores (Schiebe et al., 2012; Zas et al., 2011; Zhao et al., 2011).

Induced defences are considered a cost saving strategy because they are produced only when needed (Karban and Baldwin, 1997). However, once triggered, the production of induced defences is also costly and requires large amounts of carbohydrates (Cipollini and Heil, 2010; Sampedro et al., 2011a). Due to resource limiting conditions imposed by water, light and nutrient availability in nature, young pine trees have to optimize the allocation of resources to constitutive defences across heterogeneous environments (Moreira et al., 2014). Resource availability may also affect the allocation to induced defences, but evidence in this case is inconclusive, especially in long-lived trees (Eyles et al., 2010). For instance, some studies found greater inducibility of defences under growth-limiting conditions (Najar et al., 2014; Sampedro et al., 2011a), while others found the opposite (Lombardero et al., 2000). In order to understand this apparent inconsistency, and given that investment in defensive chemistry directly depends on carbon availability, the origin of the carbon-resources

used for producing induced defences is worth considering, i.e. to what extent do carbon-resources used in induced defences come from current photosynthates, and to what extent can they be built upon stored carbohydrates (Ruan et al., 2013). Recently assimilated carbon is known to be a major source for terpenoid biosynthesis (Gershenzon, 1994). There is a close link between CO₂ uptake and biosynthesis of terpenoids in conifer foliage, where ¹³C-labeled monoterpenes quickly appear after spiking young pines with ¹³CO₂ (Ghirardo et al., 2010). Up to 58% of the monoterpene emission was *de novo* synthesized from current assimilated carbon in *P. sylvestris* saplings (Ghirardo et al., 2010 and references therein), although alternative carbon sources (stored non-structural carbohydrates and recycled carbon) may also contribute (Ghirardo et al., 2011). Interestingly, starch grains and other carbon storage molecules found in phloem parenchyma may be available for induced synthesis of new phenolic compounds and also for the synthesis of resin in the epithelial cells of the resin canals (Krekling et al., 2000).

Furthermore, even when the carbon for chemical defences comes primarily from photosynthetic assimilation, carbon sources for oleoresin and phenolics could differ as the initial substrates required for the synthesis of those compounds differ (Schultz et al., 2013). Both types of compounds are synthesized through well differentiated pathways. Phenolic compounds are synthesized through the phenylpropanoid pathway from precursors of aromatic amino acids (Eyles et al., 2010; Moore et al., 2014), *whereas terpenoids are first built from isopentyl pyrophosphate and then converted to dimethylallyl pyrophosphate via the mevalonate and methylerythritol pathway* (Bohlmann et al., 1998; Eyles et al., 2010; Moore et al., 2014). The relative contribution of current photosynthates and stored carbon pools to plant respiration, biomass allocation, soil transfer processes and whole tree carbon balance have received

significant attention in recent years (e.g. Epron et al., 2012; Högberg et al., 2008; Kuptz et al., 2011). It is also known that C allocation patterns and within-plant mobilization of C resources may change immediately following biotic damage signalling in *Arabidopsis* (Ferrieri et al., 2013), tomato (Gómez et al., 2010; Hanik et al., 2010), tobacco (Machado et al., 2013) and poplar (Arnold et al., 2004). There is also evidence that jasmonates, a family of plant hormones deeply involved in defensive responses, could be also regulators of sugar catabolism in tobacco, thus interfering both with the allocation of carbon resources and with tissue quality and palatability (Ferrieri et al., 2015; Machado et al., 2015). All these processes may contribute to the drastic changes in biomass allocation found in young pine trees in response to herbivory signalling (Moreira et al., 2012b). However there is still poor knowledge about the extent to which allocation priorities of stored and recently-fixed carbon to chemical defences are plastic in response to biotic stress. Particularly in long-lived woody plants, large non-structural carbohydrate pools in roots and stems may be crucial for physiological processes under stressful situations (Appel et al., 2012; Najjar et al., 2014).

The aim of the present study is to determine the extent to which the production of induced chemical defences in pine trees in response to herbivory depends on the availability of current photosynthates or stored C. To this end, we exposed 2 years-old pine trees of three pine species to real herbivory by a harmful bark-chewer insect, under conditions of either natural sunlight or complete light deprivation. We measured in a time-course design the concentration of the main chemical defences (non-volatile resin and total polyphenolics) and explored whether light deprivation conditioned the increase of these defences in response to weevil damage. If induced defences are built mainly upon current photoassimilates, then light deprivation should strongly hamper the production of new defences because of carbon starvation. However, if stored carbon is

the main source for the synthesis of new induced defences, the effect of light limitation on their production would be less relevant. Besides, induced synthesis of both terpenoids and phenolics could be differentially affected as their carbon sources divert from different biosynthesis pathways.

2. Material and methods

2.1. Experimental design

Maritime pine (*Pinus pinaster* Ait.), Scots pine (*P. sylvestris* L.) and Monterey pine (*P. radiata* D. Don) were subjected to experimental herbivory by the pine weevil (*Hylobius abietis* L.) under two light treatments (complete light deprivation and natural sunlight as a control) during a one-week time-course experiment with destructive sampling. The experiment followed a randomized split-plot design replicated in 8 blocks, with light deprivation (two levels: light deprivation, 'dark' and natural sunlight, 'light') as the whole factor, and time exposure to the pine weevil (Time, four levels: 0 (constitutive), 1.5, 3.5 and 7 days) as the split factor. In total, we used 189 plants, corresponding to 8 blocks $\times$ 2 light treatments $\times$ 4 times of exposure to the pine weevil = 64 plants per pine species (61 in the case of Maritime pine due to lack of enough plant material). Additionally, a 'dark' treatment without weevils was applied to 8 extra plants of each species in order to check for potential side effects of the dark treatment on defensive allocation.

2.2. Plant material, insect rearing and plant culture conditions

Seed sources for Maritime pine, Monterey pine and Scots pine were Massif Landais (France), Astur-Cantabro provenance (Coastal North Spain) and Sierra de Guadarrama provenance (Central Spain Mountains), respectively. Maritime pine is naturally present

in southern Europe and northern Africa around the Mediterranean basin (Richardson and Rundel, 1998). Scots pine is the most widespread Eurasian pine, extending southwards from boreal areas to the Atlantic and central European forest regions reaching the southern limit of its range in southern Spain (Richardson and Rundel, 1998). Monterey pine is native to California but it is the most widely planted exotic conifer in the world, mainly in the southern hemisphere. In Europe it is widespread in the coastal region of northern Spain and in southern France (Lavery and Mead, 1998). These three pine species are among the most economically-important forest tree species in Southern Europe, where they share many native insect herbivores. Among them, the pine weevil stands out as a voracious consumer of conifer seedlings that hampers natural and artificial regeneration of all the three pine species (Zas et al., 2014 and references therein).

The pine weevil, although also uses other species as food sources, intensively feeds on the bark and phloem of several conifer species, including those in this study. This weevil is widely distributed throughout Eurasia, where by feeding on the stem bark of young trees often causes extensive mortality in plantations that can result in major economic losses (Nordlander et al., 2011). This insect pest has been widely shown to induce defensive responses in pine trees in just a few hours after damage (Moreira et al., 2013; Sampedro et al., 2011b; Wainhouse et al., 2005; Zas et al., 2011). Induced defences after weevil damage or after methyl jasmonate application -a phytohormone that mimics herbivore-induced responses (e.g. Miller et al., 2005)- substantially reduced further damage caused by the weevil (Heijari et al., 2005; Sampedro et al., 2011b; Zas et al., 2014). For the present study, adult weevils were collected at a clear-felled pine forest in Monte Castrove (Pontevedra, Spain, 42° 27' 23'' N, 8° 43' 19'' W) using Nordlander traps (Nordlander, 1987) and maintained in culture chambers at 10 °C until

the start of the experiment. During this period weevils were fed weekly with fresh twigs of *Picea abies* (L.) H. Karst.

In June 2011, six month old pines were transplanted to 2 L pots with peat and perlite (8:2) and 12 g of a slow release fertilizer (Granum, Soaga SL, Vilanova de Arousa, Spain; NPK 11-22-9), and grown outdoors at NORFOR Forestry Nurseries (Pontevedra, Galicia, Spain; 42°22'49''N 8°38'47''W) using commercial practices in a randomized spatial design. In June 2012, plants were moved to a greenhouse chamber at the Misión Biológica de Galicia - CSIC (Pontevedra, Spain) and maintained under controlled temperature conditions (14°C night, 24°C day, watering every 3-4 days) and natural daylight.

2.3. Herbivory and light deprivation treatments

In April 2013 a transparent plastic tube (100 cm tall and 12-14 cm in diameter) was fitted around each tree and light deprivation treatments were applied. Plants were randomly allotted to the corresponding sampling time and then distributed in a randomized block design in the greenhouse. Then one male and one female weevil were confined into each tube and allowed to feed on the pines for 0, 1.5, 3.5 or 7 days. Weevils were previously starved for 24 h in Petri dishes with moist filter paper at 20 °C and then individually weighed. Tubes were built from transparent polycarbonate sheets rolled on their longest side and pasted with double-sided sticky tape. A fine-mesh was attached at the top of each tube to prevent the insects from escaping. Average plant height (mean $\pm$ S.E.) at the beginning of the experiment was 63.37 ± 1.58 cm, 60.91 ± 1.31 cm, and 61.96 ± 1.61 cm for Maritime pine, Scots pine and Monterey pine, respectively.

Control plants under natural sunlight ('light' treatment) remained during the experiment covered with the transparent tube, whereas the light deprivation treatment was applied by covering the tube with a 40 µm thick black LDPE bag (Celea SA, Vizcaya, Spain; 110 x 65 cm). That black bag reduced PAR irradiance at midday from 564 ± 27 in the control plants under natural sunlight to $8 \pm 1 \mu\text{mol m}^{-2} \text{s}^{-1}$ (mean $\pm$ SE; N = 10; QMSW-SS Quantum meter, Apogee Instruments Inc., Logan, UT, USA). We checked that shading treatments did not affect temperature inside the experimental chambers by recording the temperature every 10 min during three consecutive days with HOBO Pendant ® dataloggers pending at mid-height of 3 experimental plants per treatment. Mean temperatures in the light and dark treatments were 19.9 ± 0.20 and 19.5 ± 0.20 during the day (08:00 to 21:00) and 10.90 ± 0.03 and 10.89 ± 0.03 during the night time (mean $\pm$ s.e.; N = 3). Divergence between light and dark treatments in mean, minimum and maximum daily temperatures was negligible (0.4, 0.2 and 2.2 °C, respectively).

2.4. Sampling, measurements and chemical analyses

After 0, 1.5, 3.5 and 7 days of exposure to weevil herbivory, pine weevils were removed and pine trees were destructively harvested by cutting the stem just above ground level. The damage produced by the pine weevil was measured immediately in the entire plant as debarked area (mm²) using calibrated area templates as in Nordlander et al. (2011).

Plants were then transported in ice coolers to the lab where stems were divided into 3 parts of the same length ('lower', 'middle' and 'upper') that were individually weighed (fresh weight). All material was immediately frozen and preserved at -30°C until analysis. Concentration of non-volatile resin and total polyphenolics were determined in each stem section as they are known to show marked allocation patterns

along the stem of the tree (Moreira et al., 2012a). Whole stem concentration for each chemical trait was calculated after weighing the concentration in each stem part by the mass contribution of that part in relation to the total stem biomass of that individual plant.

Resin ducts form an interconnected network and resin stored in the wood resin ducts can be also relevant for pine resistance when outer resin ducts are exposed by wounding (Krokene, 2016). Thus, the concentration of non-volatile resin was measured in the whole tissue of each stem section (including both wood and bark) as described in Sampedro et al. (2011a). Briefly, in a stem subsample of ca. 7 g fresh weight, bark tissue (i.e. phloem and cortex) was carefully separated from the wood (xylem) using a surgical knife, the wood cut in small pieces with a surgical knife, both tissues transferred together into borosilicate test tubes, and resin extracted with 10 mL of hexane (20 min at 20°C in an ultrasonic bath, and then for 24 h at room temperature). The extract was transferred carefully into new pre-weighed test tubes and the extraction step was repeated again. The solvent in the tubes was evaporated to dryness and the mass of the non-volatile resin residue was determined to the nearest 0.0001 g. Values were expressed as mg of non-volatile resin g⁻¹ stem dry weight (d.w.). This gravimetric determination of non-volatile resin in the stem was highly correlated with the concentration of the diterpenoid fraction ($r = 0.9214$; $P = 0.00002$), as quantified by gas chromatography in previous trials (Sampedro et al., 2011a). Previous studies have evidenced that the non-volatile resin fraction is inducible by pine weevil damage (e.g. (Moreira et al., 2013) and that its concentration is positively related to increased resistance against this and other insects (Sampedro et al., 2011b; Zas et al., 2014). As the non-volatile fraction represents more than the 50% of the pine oleoresin (Keeling and Bohlmann, 2006), it is a good proxy for quantitative allocation to terpene defences.

After resin extraction, total polyphenolics were analysed only in the bark tissue, as it is known that specialized cells in phloem parenchyma are the site of synthesis and storage of phenolics in conifers (Franceschi et al., 2005). Total polyphenolics were extracted with methanol and analysed colorimetrically as described in Sampedro et al. (2011a). Briefly, about 800 mg of oven-dried plant material (45°C to constant weight) was finely ground in liquid nitrogen and ca. 300 mg extracted with aqueous methanol (1:1 vol:vol) after sonication (20 min). Total polyphenolics in the extract were determined colorimetrically by the Folin-Ciocalteu assay using a Biorad 650 microplate reader (Bio-Rad Laboratories Inc., Philadelphia, PA, USA) at 740 nm, using tannic acid as standard. Concentrations were expressed as mg of tannic acid equivalents $\cdot$ g⁻¹ stem d.w.

2.5. Statistical analyses

The effects of the main design factors on weevil feeding and on the concentration of non-volatile resin and total polyphenolics in the whole stem were analysed for each species separately. Two-way mixed models were fitted with the PROC-MIXED procedure of the SAS system (Littell et al., 2006). Light, Time and the interaction between Light and Time were considered fixed factors. Block (B) and B $\times$ Light interaction were considered random factors in order to analyze the main factor Light in the split-plot design with the appropriate error term (Littell et al., 2006). Weevil weight was initially included as a covariate, but finally removed because it did not improve model fitting.

Because weevil feeding can largely differ under light and darkness conditions (Pohris, 1983), we further analysed our dataset using ANCOVA analyses to test whether the light treatments altered the covariation of non-volatile resin and total

polyphenolics with weevil damage; in other words, whether the light treatments altered the ability of the young pines to increase the concentration of induced chemical defences in response to weevil feeding. The ANCOVA analyses were performed using the PROC-MIXED procedure including Light as a fixed factor, and the Debarked Area (DA) and the Light $\times$ DA interaction as fixed covariates. A significant Light $\times$ DA interaction would indicate differences in the slopes depending on the light treatment.

Heterogeneous residual variance models were fitted when residual variance significantly differed between the two light treatments. Data for debarked area and non-volatile resin were square root- and log-transformed ($\log(x+1)$), respectively, to achieve normality in the distribution of residuals. Data are shown as least square means $\pm$ SE.

3. Results

3.1. Damage produced by the pine weevil

Light deprivation significantly favoured weevil feeding activity (Table A.1 in Appendices), with consistently more debarked area (up to 1.9 times greater) under 'dark' than under 'light' conditions for the three pine species (Figure 1). The overall effect of Light $\times$ Time interaction was not significant for the 3 pine species (Table A.1). However, specific contrasts between the two light treatments at day 7 showed significant differences only for Maritime and Monterey pine, and marginally significant in the case of Scots pine ($P = 0.07$; Figure 1).

Weevils kept feeding continuously along the course of the experiment, as shown by increasing debarked area over time for all species (Figure 1). However, in Scots pine, weevil feeding rate decreased with exposure time, while in Monterey pine, weevil damage rate remained fairly constant along the experiment (Figure B.1). Weevil feeding rate also decreased with time in Maritime pine, but the effect was not significant ($P =$

0.106, Figure B.1). Accordingly, by the end of the experiment at day 7, the most damaged species was Monterey pine (Figure 1).

3.2. Induced chemical defences in response to weevil herbivory

In the absence of weevil herbivory (undamaged plants), light deprivation during 7 days did not produced significant changes in the constitutive concentration of stem resin and phenolics (Table C.1) for the three species tested (Figure C.1).

When plants were exposed to weevil herbivory, concentration of non-volatile resin in all pine species significantly increased with exposure time to weevil herbivory, although in the case of Scots pine the effect was only marginally significant (Table A.1, Figure 2a-c). In Maritime pine and Monterey pine, the herbivore-induced concentration of non-volatile resin became significantly greater than initial constitutive levels (Time 0) after 1.5 and 3.5 days of weevil feeding respectively (Figure 2a, 2c).

Light deprivation did not significantly affect the concentration of non-volatile resin in the stems of any of the studied species (Table A.1, Figure 2a-c), although slightly higher concentrations were recorded under dark conditions in Maritime pine and Monterey pine (Figure 2a, 2c). The light treatments did not affect the temporal evolution of non-volatile resin along the exposure time to the weevil (no significant $\text{Light} \times \text{Time}$ interaction, Table A.1).

Regarding the within-plant allocation of non-volatile resin, we found, as expected, significant differences between stem parts, with greater allocation of non-volatile resin in the upper part of the stems for all species (Figure D.1.a-c). All stem parts responded similarly to weevil feeding (no significant $\text{Part} \times \text{Time}$ interaction (Figure D.1.a-c), although in the case of Scots pine, non-volatile resin significantly

increased in response to weevil feeding only in the upper part of the stem (Figure E.1).
No specific stem part significantly responded to the light treatments (Figure E.1).

The concentration of total polyphenolics in the bark remained fairly constant throughout the experiment in all three pine species, with no significant effects of the time of exposure to the weevil (Table A.1, Figure 2d-f), except for marginally so in Maritime pine where total polyphenolics tended to increase with exposure time to the weevil (Table A.1, Figure 2d). Light deprivation caused an overall increase in the concentration of total polyphenolics in Maritime pine and Scots pine, but no significant effects were observed in Monterey pine (Table A.1, Figure 2.d-f). In Maritime pine light deprivation established significant and quite large differences in the concentration of total polyphenolics between day 3.5 and the end of the experiment (Fig 2.d) whereas in Scots pine it was significant only in the middle of the experimental period (Fig 2.e). The interaction between light treatments and time of exposure to the weevils was not significant in any species (Table A.1).

Allocation of total polyphenolics significantly differed between stem parts in Maritime pine and Monterey pine but not in Scots pine (Figure D.1.d-f). In the former two species, total polyphenolics in the bark tended to increase downwards across all experimental dates (Figure D.1.a, 1c). The effect of light deprivation on total polyphenolics in Maritime pine and Scots pine was more apparent in the upper and middle parts of the stem than in the lower parts (Figure E.2). Additionally, in these two species, upper parts of the stems responded to the pine weevil damage increasing the concentration of total polyphenolics over time, but the effect was only evident under dark conditions (Figure E.2.a, 2b).

3.3. Effect of light deprivation on the production of induced chemical defences

Because weevil damage showed important differences between light treatments, and this could directly affect the intensity of the elicitation of induced defences, we conducted an ANCOVA analysis to analyze whether the ability to increase the concentration of chemical defences in response to weevil damage was altered by light deprivation (Figure 3). In the case of non-volatile resin, the ANCOVA showed that all species significantly responded to weevil damage, and produced more resin with increasing damage (all slopes were different from zero, as indicated by the significant covariation with debarked area, Figure 3.a-c). The ability to respond to weevil damage by increasing the non-volatile resin content in the stems was, however, not altered by the light treatments (no significant Light $\times$ Debarked Area interaction, Figure 3.a-c), indicating a similar slope for both 'light' and 'dark' treatments, notwithstanding a marginal effect of Light $\times$ Debarked Area interaction in Monterey pine ($F_{1,40} = 3.06$; $P = 0.088$). In the case of total polyphenolics, no significant covariation was found with weevil damage in the three pine species (all slopes not different from zero, Figure 3.d-f), and light deprivation had no effect on the covariation between weevil damage and total polyphenolics (no significant Light $\times$ Debarked Area interaction).

4. Discussion

Our results show that (i) the pine weevil fed significantly more on plants under dark conditions than under natural sunlight, and ii) the pine juveniles responded to insect feeding by increasing chemical defences proportionally to the extent of damage suffered. These two findings agree with our previous expectations (see discussion below) and enabled us to answer, after using covariance analysis, our main question (iii) whether the ability of young pine trees to produce induced chemical defences is constrained by light deprivation.

4.1. The pine weevil fed more on pines under dark conditions

Weevil feeding activity was more intense in light deprived plants than on control plants exposed to natural sunlight (Figure 1). The influence of light on the pine weevil feeding behaviour has already been studied in *Picea abies* by Pohris (1983), who found two-fold greater wounding area on seedlings after 31 h of exposure to the weevil under constant darkness than under constant full light regimes. Accordingly, a more intense feeding behaviour of pine weevil at night-time than during the day-time was found in laboratory chamber observations (Fedderwitz et al., 2014). In our study, feeding damage on the three assayed pine species was about twice as intense under light deprivation compared to control plants exposed to natural sunlight. Provided that the photoperiod during our experiment was ca. 12 h of sunlight, we could speculate that feeding activity was likely to be very low during the day-time in ‘light’ plants. As temperature was similar in both treatments, and thus not likely a driver of differential weevil behaviour, these results provide a clear quantitative confirmation of the influence of light on weevil feeding activity on all three species. However, with our experimental approach we cannot clarify whether the observed differences are purely due to feeding behaviour preferences (for instance, avoiding feeding during sunlight in order to reduce predation risks, Björklund, 2008), i.e. a top-down control, or whether interference of circadian and dark-light cycles of plant physiological regulation on defensive machinery could also play a role as a bottom-up control of herbivory (Ballaré, 2014; Goodspeed et al., 2012; Shiojiri et al., 2006). For instance, an experimental reduction of light availability in tobacco promoted depletion of fructose and glucose (but not in starch and sucrose), and favoured caterpillar weight gain, likely mediated by a diurnal, jasmonate-dependent inhibition of invertase activity (Ferrieri et al., 2015; Machado et al., 2015).

Debarked area by weevil feeding continuously increased across time, but different patterns of weevil activity were observed depending on the pine species. The feeding curve showed an asymptotic trend in Maritime pine and Scots pine (Figure 1), with a progressive reduction of the weevil feeding rate over time (Figure B.1). In Monterey pine, however, weevil feeding rate was not reduced and remained constant throughout the experiment (Figure B.1). Even when Monterey pine responded to the weevil damage by producing large amounts of induced resin, the quantitative increase in these chemical defences did not deter the feeding rate of the weevil, as they did in the two other species. Maritime pine and Scots pine are species native to Europe, which have evolved in presence of the pine weevil, whereas Monterey pine is native to North America and has not. Previous research has reported the high susceptibility of Monterey pine to native European forest pests such as the pine weevil (Zas et al., 2011; Zas et al., 2014) and the pine processionary moth (Lombardero et al., 2013). Furthermore, patterns of qualitative and quantitative allocation to induced defences in response to damage by the pine weevil differed between Monterey pine and Maritime pine (Moreira et al., 2013). In this context, our results agree with previous findings and suggest that, even when damage recognition is rapid in the non-native species, there is likely to be a lack of effectiveness of the induced chemical defensive repertoire produced in response to weevil damage.

4.2. Pine juveniles increased chemical defences in response to weevil damage

Young pines increased their whole stem oleoresin content in response to weevil damage, reaching 60% greater concentration after 7 days of real herbivory than in undamaged initial control plants. Besides, we found no direct effect of covering undamaged plants with plastic tubing on the concentration of stem oleoresin, providing

support that such an increase was driven by weevil feeding (Appendix C). Induced production of resin has been shown to be relevant increasing conifer resistance against bark beetles and fungi in field and in greenhouse conditions (Erbilgin et al., 2006; Moreira et al., 2009; Sampedro et al., 2011a; Schiebe et al., 2012; Wainhouse et al., 2005; Zhao et al., 2011). Our results agree with previous research reporting that young pines are highly responsive to pine weevil feeding, with large increases in concentration of non-volatile resin in just hours or days after attack (Moreira et al., 2013; Sampedro et al., 2011b). This indicates that the process of damage recognition and subsequent activation of resin-based induced defences is rapidly achieved.

Regarding total polyphenolics, results indicate no clear quantitative response to weevil feeding in the three pine species (Figure 2), although a slight increase in the concentration of total polyphenolics was observed in the upper part of the stem of Maritime pine and Scots pines (Figure E.2). These results agree with previous findings reporting a marginally significant increase in total polyphenolics in the stems of Maritime pine in response to weevil feeding, but no effects in Monterey pine (Moreira et al., 2013).

As previously observed in Monterey pine (Moreira et al., 2012a), chemical defences showed marked within-plant allocation patterns, with non-volatile resin increasing upwards and total polyphenolics increasing downwards across pine height (Figure D.1). Additionally, we found that the stem net increase in oleoresin resulted from a differential response to damage across the low, medium and upper stems parts of the plants. Induced increase in oleoresin content was significant for the whole stem in Maritime pine and Monterey pine, but in Scots pine just the upper part of the plant responded to weevil damage in this way (Figure D.1.a-c). These results agree with previous research reporting that young pines show a strategy of differential allocation of

chemical defences, where the upper parts are the more plastic and show the greatest increase in resin concentration after herbivore damage (Moreira et al., 2012a), although this pattern shows some variation across species (Sampedro et al., unpublished results). The quantitative approach used for whole-stem analysis of chemical defences, with a stratified strategy for tissue sampling and chemical analyses, was a good methodological approach that highlighted the marked changes in concentration of chemical defences across upper, mid and lower plant parts.

4.3. Light deprivation did not constrain the production of induced chemical defences

The main finding of our study was that light deprivation during 7 days did not significantly constrain the capability of young pine trees for *de novo* synthesis of oleoresin defences in response to insect herbivory. It is methodologically challenging to quantify the effect of experimental treatments on the responsiveness of plants to herbivory damage. Induced responses vary with insect feeding intensity, which in turn can be affected by the experimental treatments, so both the direct effect of the treatments on inducibility of chemical defences and the indirect effect mediated by alterations of the insect damage across treatments are confounded. This situation becomes trickier when the target plant tissue (in our case, the main stem on which weevils feed) cannot be sampled without complete plant harvesting. To address these challenges, we accounted for the likely influence of light regimes on weevil feeding behaviour by using an ANCOVA design for testing our hypothesis, analyzing covariation of whole-stem production of chemical defences with weevil damage under control and light-deprivation conditions. Using this ANCOVA approach, we found that resin production covaried with weevil damage for all the three studied species. Interestingly, however, such covariation was not significantly influenced by light

regime; in other words, and in answer to our main question, light deprivation did not impair the plants' ability to produce induced stem oleoresin.

The biosynthesis of terpenoids is metabolically costly and it has been estimated at 3.5 and 3.2 g of glucose equivalents per gram of typical mono- and diterpenes respectively (Gershenzon, 1994). Terpenoids are reduced carbon compounds that need highly specific enzymatic machinery, and also require expensive specialized storage structures (Gershenzon, 1994). Furthermore, all carbon invested in oleoresin is definitively sequestered and unavailable for other uses, as terpenoids cannot be further catabolised back into usable carbon compounds (Gershenzon, 1994). Polyphenolic compounds, which are also expensive carbon compounds, are synthesized and stored in pine trees in high concentrations in vacuolar inclusions in the phloem parenchyma cells in the secondary phloem (Franceschi et al., 1998). Unlike terpenoids, however, they may be recycled into more simple carbon compounds and used for other functions (Li et al., 2012). Early studies have reported that synthesis of monoterpenes appear to be light dependent in conifers (Gleizes et al., 1980; Gref and Tenow, 1987) and other plants (Zavala and Ravetta, 2001). More interestingly, inducibility of monoterpene synthase activity in response to wounding appeared to be severely compromised in conditions of carbon starvation (either light deprivation or severe water stress) (Lewinsohn et al., 1993). However, in the present study we did not find evidence that the absence of C assimilation constrained the ability of young pines to induce the accumulation of diterpenoid chemical defences. Monoterpenes and diterpenes could be thus differentially regulated by light deprivation.

By evaluating chemical defences in the whole stem, and restricting the synthesis of photosynthates by complete light deprivation, we can infer that young pines were able to use other carbon pools than current photosynthates, likely stored carbohydrates, for

de novo synthesis of diterpenes in response to insect herbivory. A significant net increment in the stem content of non-volatile oleoresin defences in the absence of C assimilation was found in all three species. This suggests a likely relevant role of carbon remobilization from storage carbohydrates in *de novo* biosynthesis of resin- (and also phenolic-) induced chemical defences in young pines. These results were particularly noteworthy for non-volatile resin for which the concentration in the stems clearly increased in response to insect damage

Induction of non-volatile resin and total polyphenolics are known to impose differential costs in young pine trees. For instance, inducibility of total polyphenolics in young Maritime pine showed a negative genetic correlation with growth potential (Sampedro et al., 2011a), while no significant correlation was observed in the case of inducibility of non-volatile resin. Assuming that carbon sources for growth are mainly derived from current photosynthates, these results agree with those found here, in which inducibility of non-volatile resin does not depend on current photosynthates. Additionally, results from Sampedro et al. (2011a) also indicated that concentration of constitutive (but not of induced) resin in stems of young Maritime pine was negatively correlated with growth potential, suggesting that the concentration of constitutive and induced oleoresin may use different carbon sources, or at least evidencing some plasticity in carbon allocation after herbivore signalling. Accordingly, our results do not exclude the possibility of a flexible and plastic allocation of carbon resources to induced chemical defences. For instance, current assimilated carbon could be used for the production of induced defences under regular conditions, but stored carbon pools may be mobilized upon light or water deprivation. This and other related questions must be tested by further research using isotope labelling of carbon pools.

The question of revealing the origin of carbon resources that pine trees use for synthesizing chemical defences is crucial for understanding the [integration of plant](#) responses to multiple stressors. For instance, under water stress conditions, isohydric species such as Mediterranean pine trees rapidly close stomata to avoid water loss, at the same time limiting the supply of fresh carbon assimilates, and leading to carbon starvation if the water stress period extends for too long (Sala et al., 2012). Thus, it is important to understand the plasticity and flexibility in C allocation, and to disentangle the C sources used for the synthesis of wound-induced resin and phenolics, in order to predict resistance and tolerance to herbivory under scenarios of abiotic stress in a changing environment.

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Author contributions: RZ and LS conceived and designed the experiment; RZ, LS and XLG performed the experiment, carried out the chemical analyses and [analysed](#) the data; XLG wrote the first draft and all authors contributed to revisions and writing of subsequent versions.

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FIGURE CAPTIONS

Figure 1. Damage by the pine weevil over time in three pine species (*Pinus pinaster*, *P. sylvestris* and *P. radiata*) exposed to pine weevil feeding under natural sunlight ('light') and complete light deprivation ('dark'). Trees were two-year old. *P* values in the companion tables indicate the significance of the main effects. Significant *P* values ($P < 0.05$) are typed in bold. Asterisks within figures indicate significant differences between light and dark conditions in each Time of exposure (*: $P < 0.05$; **: $P < 0.01$). Each point represents the mean $\pm$ SE; N = 7 or 8 replicate plants.

Figure 2. Concentration of non-volatile resin (upper panels) and total polyphenolics (lower panels) over time in three pine species (*Pinus pinaster*, *P. sylvestris* and *P. radiata*) exposed to pine weevil feeding under natural sunlight ('light') and complete light deprivation ('dark'). Trees were two-year old. *P* values in the companion tables indicate the significance of the main effects. Significant *P* values ($P < 0.05$) are typed in bold. Different letters within the panels indicate significant differences between times of exposure to the weevil ($P < 0.05$). Asterisks indicate significant differences between light and dark conditions at each specific Time (*: $P < 0.05$; **: $P < 0.01$). Each point represents the mean $\pm$ SE; N = 7 or 8 replicate plants.

Figure 3. Relationship between the pine weevil damage (measured as cumulative debarked area) and the concentration of non-volatile resin (upper panels) and total polyphenolics (lower panels) in three pine species (*Pinus pinaster*, *P. sylvestris* and *P. radiata*) exposed to pine weevil feeding under natural sunlight ('light') and complete light deprivation ('dark'). Trees were two-year old. Each point represents a single plant. The companion tables include the *P* values associated with the ANCOVA analysis

testing for differences in intercepts (Light effect), for a common covariation with weevil damage across light treatments (Debarked Area, DA) and for differences in the slopes between light treatments (Light $\times$ DA). Significant P values ($P < 0.05$) are typed in bold. A single regression line was included for both light treatments because of no significant Light $\times$ DA interaction.

Figure 1. Lopez-Goldar et al.

Figure 2. Lopez-Goldar et al.

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Figure 3. Lopez-Goldar et al.

Appendices

[Carbon starvation by light deprivation does not constrain the ability of young pines to produce induced chemical defences in response to a bark-chewing herbivore,

by López-Goldar, X.; Sampedro, L.; Zas, R.]

Appendix A. Summary of the results from the mixed models

Table A.1. Summary of the effects of Light deprivation, Time of exposure to real herbivory by the pine weevil and their interaction on weevil damage (Debarked area), and on the concentration of chemical defences (non-volatile resin and total polyphenolics) in stem tissues of three pine species. Two-years old pine trees were exposed to real herbivory during 7 days and destructively sampled in a time-course design. Degrees of freedom (DF), *F*-ratios and *P*-values are shown. Significant *P* values ($P < 0.05$) are typed in bold.

	<i>P. pinaster</i>			<i>P. sylvestris</i>			<i>P. radiata</i>		
	DF (n,d)	F	<i>P</i>	DF (n,d)	F	<i>P</i>	DF (n,d)	F	<i>P</i>
<i>Debarked area</i>									
Light deprivation	1, 7	9.34	0.018	1, 7	6.52	0.038	1, 7	12.34	0.010
Time of exposure	2, 23	5.07	0.015	2, 27	6.83	0.004	2, 24	28.86	<0.001
Light × Time	2, 23	0.42	0.663	2, 27	0.16	0.856	2, 24	0.41	0.665
<i>Non-volatile resin</i>									
Light deprivation	1, 7	3.97	0.087	1, 7	0	0.988	1, 7	2.15	0.186
Time of exposure	2, 26	7.45	0.003	2, 28	2.67	0.087	2, 28	14.56	<0.001
Light × Time	2, 26	0.68	0.516	2, 28	0.97	0.393	2, 28	0.91	0.414
<i>Total polyphenolics</i>									
Light deprivation	1, 4	9.28	0.038	1, 4	8.27	0.045	1, 4	0.99	0.377
Time of exposure	2, 16	2.76	0.093	2, 16	1.43	0.269	2, 16	0.95	0.405
Light × Time	2, 16	0.3	0.747	2, 16	0.81	0.461	2, 16	0.25	0.785

Appendix B. Temporal dynamics of weevil feeding rate

Figure B.1. Feeding rate (debarked area inflicted per day) by the pine weevil over time in three pine species (*Pinus pinaster*, *P. sylvestris* and *P. radiata*) exposed to pine weevil feeding under natural sunlight (‘light’) and complete light deprivation (‘dark’). Trees were two-year old. *P* values in the companion tables indicate the significance of the main effects. Significant *P* values (*P* < 0.05) are typed in bold. Each point represents the mean ± SE; N = 7 or 8 replicate plants.

Appendix C. Effect of light deprivation on the concentration of chemical defences in undamaged plants.

In order to test whether the light deprivation treatment (a polyethylene black bag covering the acrylic tube around the plant) could generate any undesired side effect on defence allocation in the plants by itself, without weevil feeding, we allotted 8 plants of each pine species to the dark treatment without exposition to the insect. Those plants were destructively sampled at time 0 days (initials) and 7 days and non-volatile resin and total phenolics determined in the stem.

Concentration of chemical defences in the tree conifer species did not significantly change after seven days of light deprivation. The lack of a direct effect of covering the plants with a plastic tube during 7 days on the concentration of chemical defences supports that the changes observed in the main experiment were mainly in response to weevil feeding.

Fig C.1. Concentration of non volatile resin and total phenolics in the whole stem of three pine species (*Pinus pinaster*, *P. sylvestris* and *P. radiata*) before and after seven days of complete light deprivation. The effect of shading did not significantly affect the concentration of chemical defences in the stem (see also Table F1). Shading treatment consisted of a black plastic polyethylene bag covering the whole plant promoting complete darkness. Trees were two-year old. Means ± se. N = 5 to 8 plants.

Table C.1. Results of ANOVA analyzing the effect of seven days of complete light deprivation (two levels: dark and light) on the concentration of quantitative chemical defences in the stem of young pines of three species. Shading did not significantly affect the concentration of chemical defences in the stem regarding light plants. Shading treatment consisted of a black plastic polyethylene bag covering the whole plant promoting complete darkness.

Pine species	Chemical defences			
	Non-volatile resin		Total Phenolics	
	F ratio	P	F ratio	P
<i>Pinus sylvestris</i>	$F_{1,14} = 1.35$	0.2634	$F_{1,9} = 0.47$	0.4419
<i>Pinus radiata</i>	$F_{1,14} = 0.849$	0.3723	$F_{1,9} = 0.949$	0.3553
<i>Pinus pinaster</i>	$F_{1,13} = 2.58$	0.1319	$F_{1,9} = 0.009$	0.9280

Appendix D. Within plant allocation of chemical defences

For supporting the evidence of differential allocation of defences across the different plant parts of pine juveniles, as previously demonstrated by Moreira et al. (2012a), non-volatile resin and total polyphenolics in each stem section were analysed with a repeated measures mixed model. The analyses, which were done separately for light and dark conditions, included Time as a between-subject fixed factor, and Part and Part $\times$ Time as within-subject fixed factors. Block and Block $\times$ Time were included in the model as random factors. The results for plants under natural sunlight conditions are shown here.

Figure D.1. Concentration of non-volatile resin (upper panels) and total polyphenolics (lower panels) over time in each stem part (Lower, open symbols; Middle, grey

symbols; Upper, black symbols) in three pine species (*Pinus pinaster*, *P. sylvestris* and *P. radiata*) exposed to feeding by the pine weevil under natural sunlight. Trees were two-year old. Each point represents the mean $\pm$ SE; N = 7 or 8 replicate plants. P values in the companion tables indicate the significance of the main effects. Significant P values ($P < 0.05$) are typed in bold.

Appendix E. Effect of light deprivation on the within-plant variation in the concentration of chemical defences.

Figure E.1. Concentration of non-volatile resin in the upper (a, b, c panels), middle (d, e, f panels) and lower (g, h, i panels) stem parts of three pine species (*Pinus pinaster*, *P. sylvestris* and *P. radiata*) exposed to feeding by the pine weevil under natural sunlight ('light') and complete light deprivation ('dark'). Trees were two-year old. Each point represents the mean $\pm$ SE; N = 7 or 8 replicate plants. P values in the companion tables indicate significance of the main effects. Significant P values ($P < 0.05$) are typed in bold.

1024 **Appendix F. Media file with a quick video summary of the paper.**

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1026 <https://www.youtube.com/watch?v=8X2oF5W2cJE>

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video summary of the paper

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