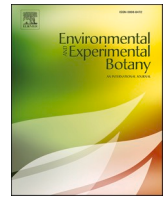




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Warming alters sex-specific responses in leaf defense against insect herbivory in *Populus cathayana*

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ABSTRACT

Climate warming has been found to increase forest insect herbivory with potentially important consequences for the performance and distribution of plant species. Dioecious species often show sex-specific responses to abiotic drivers leading to spatial segregation of the sexes. However, little is known about sexual differences in defense against herbivorous insects in response to warming. We used a field survey and glasshouse experiment to test how sex-specific defenses of *Populus cathayana* against insect herbivory respond to warming. Consumed leaf area in females was greater than in males at +4 °C experimental warming or lower altitude. The warming treatment did not strongly affect differences in constitutive levels of leaf salicortin, condensed tannins (CT), salicylic acid (SA) and jasmonic acid (JA) between the two sexes. However, warming decreased herbivore-induced responses in salicortin, CT, SA, JA and defensive-related enzyme activities (e.g., superoxide dismutase, peroxidase, polyphenol oxidase and lipoxygenase) more in females than in males. Moreover, consumed leaf area in females showed more negative relationships with feeding-induced chemical defense traits than in males. These results show that sex-specific leaf defense against insect herbivory can be mediated by climate warming, and that higher leaf herbivory in females under warming is mainly influenced by decreases in induced defense responses rather than constitutive defense responses.

1. Introduction

Warming associated with global climate change are expected to increase the frequency and severity of insect outbreaks (Haynes et al., 2014; Deutsch et al., 2018; Pureswaran et al., 2018). Insects' outbreaks under climate change are major agents of forest ecosystem change worldwide (Pureswaran et al., 2018). In recent decades, insect herbivore outbreaks driven by climate change have been tied to regional and global patterns of forest mortality, which will likely impact the distribution of plant species (McDowell et al., 2011; Haynes et al., 2014). The mechanistic link between elevated temperature and forest pest outbreaks is still unclear because both plants and herbivores may respond independently to warming and climate drivers may have synergistic or antagonistic effects on insect outbreaks (Jactel et al., 2019). As plants are immobile and form the base of many ecosystems, plant defense against ongoing outbreaks of herbivorous insects is crucial for plant fitness and the maintenance of ecosystem functions (Meldau et al., 2012;

Cope et al., 2019). Studying herbivore defenses in trees, in particular, is important for understanding forest mortality events and for forest management (McDowell et al., 2011; Jamieson et al., 2015).

Plants' main defensive strategy involves a complex network of morphology, physiology, and biochemistry (Kerchev et al., 2012; Züst and Agrawal, 2016). These networks include leaf toughness, secondary metabolic products, defensive enzymes and phytohormones (e.g., salicylic acid, jasmonic acid) (reviewed by War et al., 2012). These defenses can be divided into constitutive defenses (which are always present) and induced defenses (which are produced at or mobilized to the site where a plant is injured) (Eichenberg et al., 2015; Karban, 2020). Some studies have observed that trade-offs between constitutive and induced defenses, and the relative importance of the two defense strategies may shift along environmental gradients (Moreira et al., 2014; Rasmann et al., 2015). Induced defenses via physiological adjustments are usually less costly than the always present constitutive defenses (Karbon, 2020), suggesting that induced defenses may be more cost-effective than

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constitutive defenses in a changing world. While plants have evolved strategies to deal with herbivorous opponents over millions of years (Züst and Agrawal, 2016), our understanding of the intricate network of defenses against insect herbivory under abiotic and biotic stresses remains limited, especially how induced defenses in trees will respond under global climate change (Jamieson et al., 2017; Holopainen et al., 2018; Li et al., 2021).

In recent years, several studies have found that global warming can alter physiological and molecular defensive responses in trees (Jamieson et al., 2015, 2017). Some studies have found that warming may increase tree susceptibility to insect herbivores (DeLucia et al., 2012), which may be related to decreases in the concentrations of defensive chemicals under warmer temperatures (Jamieson et al., 2015; Holopainen et al., 2018; Sobuj et al., 2018; Forbes et al., 2020); while warming may increase defensive phytohormone concentrations such as jasmonic acid (DeLucia et al., 2012). These complex modifications in defense chemistry and physiology under warming can vary by plant species (Zvereva and Kozlov, 2006; Rasmann et al., 2014; Jamieson et al., 2015) and even with genotypes (Lindroth and St. Clair, 2013; Barker et al., 2019; Galmán et al., 2018).

Dioecious plants make up only 5–6% of all plant species (Renner, 2014), but play important ecosystem roles. For example, plants in the Salicaceae family often act as pioneer tree species, playing an important role in regulating the structure and function of forest ecosystems (Hultine et al., 2016). Because males and females have different investments in reproduction (females invest energy in flower and fruit formation, while males invest only in flowering and pollen) and different post-reproductive survival abilities, dioecious plants have been a model system for understanding resource allocation between defense and other life history stages (Barrett and Hough, 2013; Brzyski et al., 2014; Liao et al., 2020). Although females are typically more resistant to insect herbivory than males (Cornelissen and Stiling, 2005), this pattern may be reversed in some species (Buckley and Avila-Sakar, 2013; Zhang et al., 2019) or there may be no difference between sexes (Robinson et al., 2014). Environmental conditions may drive these discrepancies among species (Brzyski et al., 2014; Randriamanana et al., 2014; Chen et al., 2016; Zhang et al., 2019; Zhang et al., 2020; Liu et al., 2021). If sexual differences are environmentally context-dependent, global change and subsequent changes in resource availability may exacerbate sex-specific responses in growth and physiology, which could alter population sex ratios and thus population dynamics of dioecious species (Tognetti, 2012; Hultine et al., 2013, 2016; Petry et al., 2016; Compagnoni et al., 2017). While previous studies have focused on sex-specific adaptive responses in growth under climate change conditions, few studies have investigated sex-related differences in defensive traits in this context (*but see* Sobuj et al., 2018; Zhang et al., 2019).

Species in the *Populus* genus have been used as models to elucidate tree's adaptive responses to environmental change (Chen et al., 2009; Luo et al., 2013), including how trees respond to insect herbivore communities (Rubert-Nason et al., 2015; Sobuj et al., 2018; Barker et al., 2019). In this study, we used *Populus cathayana* Rehd., an economically and ecologically important dioecious tree species, to investigate how defense against insect herbivory in females and males changes under warming. Previous studies in *P. cathayana* have found that the growth and antioxidant enzyme activity of females are more sensitive than males to changes in abiotic (Xu et al., 2008; Zhang et al., 2012; Liao et al., 2020; Xia et al., 2020) and competitive (Chen et al., 2015; Wu et al., 2018) conditions. The rates of photosynthesis and growth of female *P. cathayana* increase more than males under moderate warming treatments (Xu et al., 2008), yet it is unclear how warming will influence sex-specific responses in defense. Here, we used natural surveys to quantify leaf herbivory on female and male *P. cathayana* along an elevational temperature gradient, and controlled experiments to test the direct impacts of temperature on sex-specific responses in leaf defense mechanisms (physical, biochemical, protective enzyme activities and phytohormones). We predicted that climate warming would decrease

foliar defenses, and that there would be a higher difference between the sexes due to reproduction-mediated changes in carbon allocation. We ask the following questions: (1) Are there sex-specific differences in levels of insect herbivory and leaf defense against herbivores? (2) Does temperature influence leaf constitutive and induced defense against insect herbivory? (3) If so, how do defense responses in physical, chemical and physiological traits differ between the two sexes under warming?

2. Materials and methods

2.1. Study species

Populus cathayana is a fast-growing, widely distributed, deciduous tree species in the subtropical and temperate northern hemisphere in Asia. The species distribution ranges from about 26 °N to 40 °N, experiencing average annual temperatures between ~7–20 °C. As *P. cathayana* plays important ecological and economic roles, including as a source of bioenergy or use in environmental remediation, preventing soil erosion, and regulating microclimate conditions. Over the past few decades, *P. cathayana* forests have experienced outbreaks of insect herbivory, resulting in declining growth, and even mortality (Xu, 2019).

2.2. Field survey

In July of 2016, we conducted a field survey of leaf herbivory on *P. cathayana* at Xiaowutai Mountains (39.78°–40.15 °N, 114.78°–115.50 °E) in Hebei province, in northern China. The average annual and growing season (May to September) temperatures at the highest site are respectively 9.2 °C and 21.5 °C (from WorldClim 2.1). In this site, *P. cathayana* is a dominant species across riparian woodlands ranging from 1350–1750 m. We marked the gender of all the adult individuals in early April (flowering period) of 2016 (Xu and Dong, 2017). In five sites at 100 m increments along one transect spanning this gradient (1350–1750 m), we randomly selected 15 female and 15 male individuals (randomly selected two branches per individual) at each elevation. In poplar forests, the main insect defoliators are Lepidoptera (such as Notodontidae, Noctuidae, Lymantriidae) and Coleoptera (Xu, 2019). For each branch, we calculated the proportion of damaged leaves by these insect herbivores out of the total number of leaves, and measured the average consumed area (mm²) for each damaged leaf using millimeter paper.

2.3. Warming experiment

On March 5, 2018 (before bud sprout), 20 healthy shoots of female/male *P. cathayana* from 30 individual mature trees (background details see Liu et al., 2020) were collected from the experimental field station of China West Normal University (30°48' N, 106°03' E; 276 m above sea level). Five similar-size cuttings (10 cm in length) from each shoot were planted in a glasshouse at China West Normal University. Mean annual temperature of this site ranges from 15.6 to 17.4 °C (the average is 22.8 °C from March to September) (from Meteorological Bureau of Nanchong (<http://sc.cma.gov.cn/ds/nc/>)). The glasshouse was open on the sides to maintain ambient light and temperature but block natural rainfall; the light transition rate (measured by LI-190R Quantum Sensor) was over 90 % and the difference of average temperature inside and outside the glasshouse was below 0.3 °C. After 60 days, 40 similarly sized cuttings (20 males and 20 females; 15 cm in length and 0.5 cm in diameter) were chosen randomly and transplanted to plastic pots (15 cm × 20 cm, 1 cutting per pot) filled with 8 kg of homogenized soil, and another 5 individuals of each sex were chosen randomly to determine their dry mass as a sample at the beginning of the experiment.

The experiment was four factorial combinations of two sexes (female and male) and two temperatures (ambient and warmed). Each treatment was replicated with 10 individuals. We elevated temperature by ~ 4.0

°C (a widely used level of warming to study the effects of warming on trees; e.g., Xu et al., 2008; Liao et al., 2020) measured from the upper middle of the canopy with infrared heat lamps placed about 1 m above each warming-treated plant canopy. The warming treatment was maintained during the growing period from May 1 to September 1, 2018. Each pot was irrigated with 500 ml of water every two days, which matched typical local soil moisture measured using a time-domain reflectometer (Dong et al., 2019).

Feeding test To test the effect of warming on physiological, insect herbivore-induced leaf defense responses in each sex, four pots from each treatment were randomly selected for insect herbivore feeding trials at the end of experiment (late growing-season). *Spodoptera litura* (Lepidoptera: Noctuidae), a leaf-chewing generalist commonly found during field surveys in local *Populus* forests was chosen as a model. Ten third-instar caterpillars of *S. litura* were released onto the apical leaves of these trees and enclosed with knitted mesh. All the caterpillars were starved for 24 h before the feeding trial. After feeding over 24 h, consumed leaf area was estimated via millimeter paper to be consistent with field survey methods. All plants were placed in ambient temperatures during feeding trials to control for the direct influence of temperature on the herbivores.

2.4. Plant harvest

At the end of experiment, four plants in each treatment that were not used in herbivore feeding trials were randomly selected to measure growth, leaf toughness, leaf dry mass per area and leaf water content. Each individual was harvested and all the leaves were divided into two parts and weighed. One part (along with the post-feeding trial leaves) was immediately placed in liquid nitrogen and stored at -80°C for later measurements of secondary metabolites, defensive enzyme activity and phytohormone concentrations; the second part was used to quickly measure leaf fresh mass, fresh area (measured by a leaf area meter Li3000C (LI-COR, Lincoln, NE, USA)) and toughness, and then oven-dried at 70 °C along with the stem and roots to a constant mass.

2.5. Plant relative growth rate, and leaf physical defense traits measurement

Plant relative growth rate (RGR, $\text{g g}^{-1} \text{d}^{-1}$) during the treatment period (Δt) was estimated as $\text{RGR} = (\ln(M_2) - \ln(M_1)) / \Delta t$, where M_1 and M_2 were the total dry mass (leaf, stem and root) at the beginning (average dry mass from 5 individuals of each sex on May 1) and end of the experiment, respectively.

Leaf toughness, an important functional trait implicated in leaf physical defense, was measured as the maximum force required to punch holes through leaves (Onoda et al., 2011). We chose three mature leaves on each individual, and measured each leaf four times. A penetrometer with a 0.5 mm diameter flat-end punch rod (Handpi, Handpi Company, Zhejiang, China) was used to penetrate the leaves, excluding the leaf midribs. Leaf dry mass per area was calculated as dry mass divided by leaf fresh area. Leaf water content was calculated as fresh mass minus dry mass divided by fresh mass. The dry mass or fresh mass weighed on an electronic balance (Sartorius, Suzhou, Jiangsu, China).

2.6. Leaf chemical metabolites measurement

Condensed tannins and salicinoid phenolic glycosides are the two principal chemical defense compounds against insect herbivory in many *Populus* species (Boeckler et al., 2011; Lindroth and St. Clair, 2013). In this study, we analyzed the concentrations of condensed tannins and salicortin (the most abundant defense compound in salicinoid phenolic glycosides). Freeze-dried leaf samples from non-feeding or post-feeding plants were homogenized in a ball mill. Condensed tannins concentration was determined using the BuOH:HCl method as described by Boecker et al. (2013). Salicortin concentration was determined using the

high-performance liquid chromatography (HPLC) method described by Massad et al. (2014). Briefly, 20 mg of freeze-dried ground leaf material was added to 1 ml of MeOH with phenyl β -D-glucopyranoside as an internal standard (0.2 mg ml^{-1}). Samples were extracted by shaking on a paint shaker for 30 min at room temperature. Samples were centrifuged and extracted samples were run on the HPLC (Agilent HP1100 Series). Individual extractions from samples were injected in the HPLC column (20 μl injection volume) with a water (solvent A) and acetonitrile (solvent B) gradient at a flow rate of 1 ml min^{-1} . Salicortin was identified based on a comparison of retention times and UV absorption spectra compared to an authentic standard, which was kindly provided by Dr. Richard L. Lindroth from the University of Wisconsin, USA. Final results were calculated from peak spectral areas relative to the internal standard at 200 nm using response factors determined from a dilution series of pure compounds. All the results are presented as $\text{mg g}^{-1} \text{DW}$.

2.7. Antioxidant enzymes activity and phytohormone measurement

Defense-related enzymes are crucial in regulating plant defense against insect herbivory by modulating physiology in response to biotic and abiotic environmental changes (War et al., 2012). We analyzed five defense-related enzymes including phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), lipoxygenase (LOX), peroxidase (POD) and superoxide dismutases (SOD) (War et al., 2012; Tang et al., 2013; Xie et al., 2017). Freeze-dried leaf samples from both non-feeding or post-feeding plants were used similarly to measurements of salicortin for measurements of defensive enzyme activity and phytohormones after being homogenized in a ball mill. Activities of PAL, LOX, POD and PPO were spectrophotometrically determined as described by Tang et al. (2013) and Xie et al. (2017). One unit of enzyme activity (U) of PAL, LOX, PPO and POD was defined as a change of 0.01 in absorbance per minute at 290 nm, 234 nm, 420 nm, 470 nm, respectively. Activity of SOD was determined as described by Zhang et al. (2012). One unit of SOD activity was defined as the amount of enzyme required to cause 50 % inhibition of the reduction of nitroblue tetrazolium when monitored at 560 nm. The soluble protein concentration was determined as described by Bradford (1976).

Leaf salicylic acid (SA) and jasmonic acid (JA) concentrations were quantified following Fabisch et al. (2019). Briefly, approximately 10 mg of flash-frozen leaves were ground to a fine powder under liquid nitrogen and then lyophilized. Each lyophilized sample was extracted with 1 ml of methanol containing 4 μl of phytohormone standard mix (SA and JA; Sigma-Aldrich). The contents were vortexed vigorously for a few seconds, incubated for 25 min at 20 °C, and then centrifuged at $13\,000 \times g$ at 4 °C for 5 min. The supernatant was transferred to new microcentrifuge tubes for analysis of phytohormones on a high performance liquid chromatography-tandem mass spectrometer (HPLC-MS/MS; Shimadzu LCMS 8040 system). The concentrations of SA and JA were determined relative to the corresponding internal standard; results are presented as $\text{ng g}^{-1} \text{DW}$.

2.8. Statistical analyses

For the field survey results, we used two-way analysis of variance (ANOVA) to test the effects of sex, altitude and their interaction on the proportion of damaged leaves and average area consumed per damaged leaf. Tukey's HSD (Honestly Significant Difference) test was used to evaluate differences among altitudes in each sex, and *t* tests were used to compare the differences between sexes at each altitude.

For the warming experiment, two-way analysis of variance (ANOVA) was used to test the effects of sex, temperature and their interaction on growth and leaf parameters (including traits of non-feeding plants, feeding plants, and the feeding-induced responses: responses = $(V_F - V_{NF}) / V_{NF} \times 100\%$, where V_F is the trait value of post-feeding plants, and V_{NF} is the average value of non-feeding plants). Following ANOVA, we used Tukey's HSD test to evaluate differences between particular

treatment groups. Simple linear regressions were used to assess the relationship between consumed area and post-feeding responses of secondary metabolites and phytohormones.

All the effects were considered significant at $P < 0.05$. All data were analyzed with the software Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA) version 22.0.

3. Results

3.1. Leaf attack rates by insect herbivores across altitude

In the field survey, we found that the proportion of damaged leaves and leaf area consumed in both sexes of *P. cathayana* significantly decreased with increasing elevation, yet this pattern was stronger in females than in males (Fig. 1a, b). The average consumed area was affected by the interaction of sex and elevation (Fig. 1b). Although there was not a significant difference between females and males in the proportion of damaged leaves at each elevation, the average consumed area of males beyond 1600 m above sea level was greater than that of females, while the opposite was true below 1400 m, and in mid-altitude sites, damage was similar between the sexes.

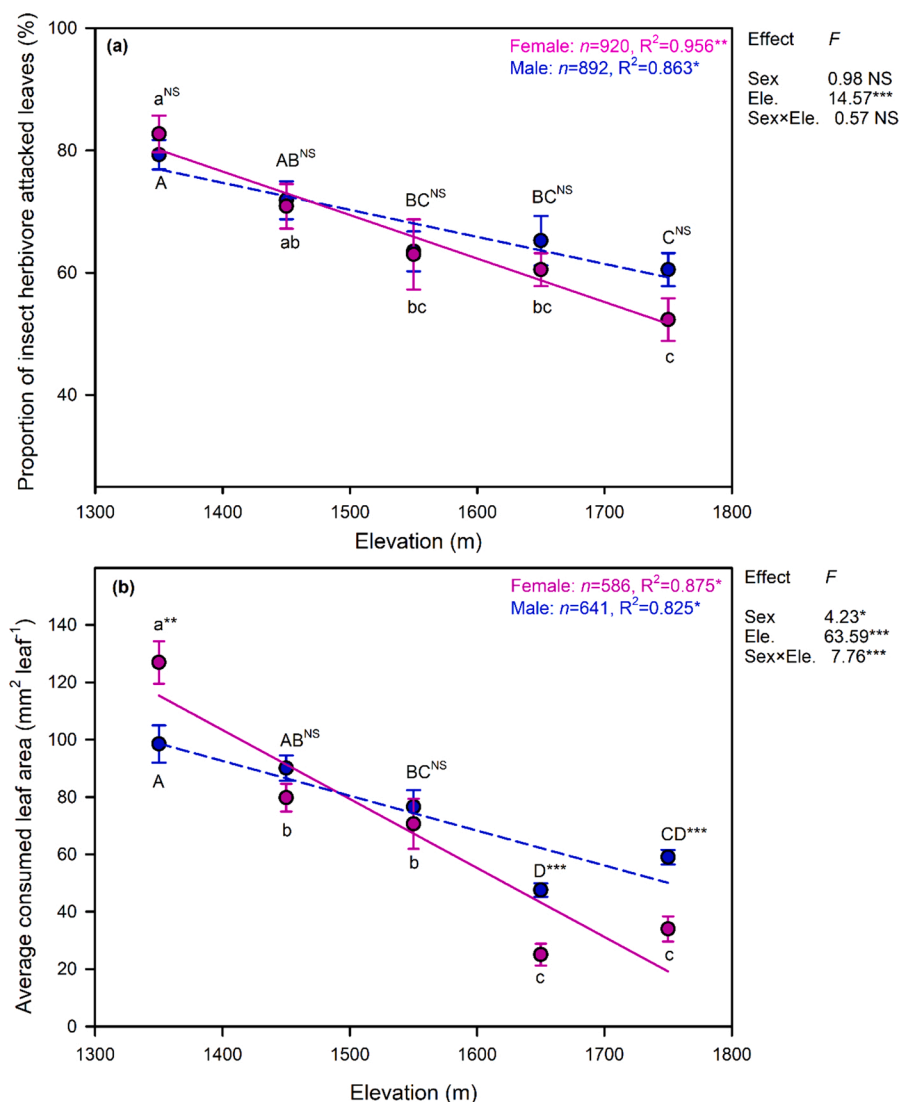


Fig. 1. Proportion of insect herbivore-attacked leaves (a) and average consumed area per attacked leaf (b) in female (pink circle; pink line) and male (blue circle; blue line) *Populus cathayana* adults along an elevation gradient. Each value is the mean \pm SE. Different uppercase/lowercase letters indicate significant differences in males/females among different elevations according to Tukey's HSD tests ($P < 0.05$). The asterisks demonstrate statistically significant differences according to independent samples *t*-test between two sexes within each elevation ($P < 0.05$). NS, $P > 0.05$; *, $P < 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$ (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.2. Leaf area consumed by *Spodoptera litura* with warming

We found that the area of *P. cathayana* consumed by the leaf-chewing generalist *Spodoptera litura* was significantly affected by sex and warming treatments (Fig. 2). Both sexes showed an increase in the area consumed with the +4°C warming treatment, but the intensity of this effect of warming on consumed area was different. Under ambient temperature conditions, the consumed area was significantly lower (48.12 %) in females than males, but was significantly higher (92.91 %) in females than males under warming ($P < 0.001$).

3.3. Sexual differences in leaf physical defense and plant relative growth rate under warming

Warming and sex both significantly influenced leaf toughness and dry mass per area (LMA) (Fig. 3), and plant relative growth rate (RGR; Fig. S1). Warming decreased RGR in both sexes. Female RGR was higher than male RGR under ambient temperatures, but was similar between the sexes under warming.

We observed higher leaf toughness in females than in males under ambient temperatures, but the sexes had similar leaf toughness under warming (Fig. 3a). While LMA was more similar between both sexes under each temperature condition, warming increased leaf toughness and LMA in males more than that females (Fig. 3a, b). Overall, leaf

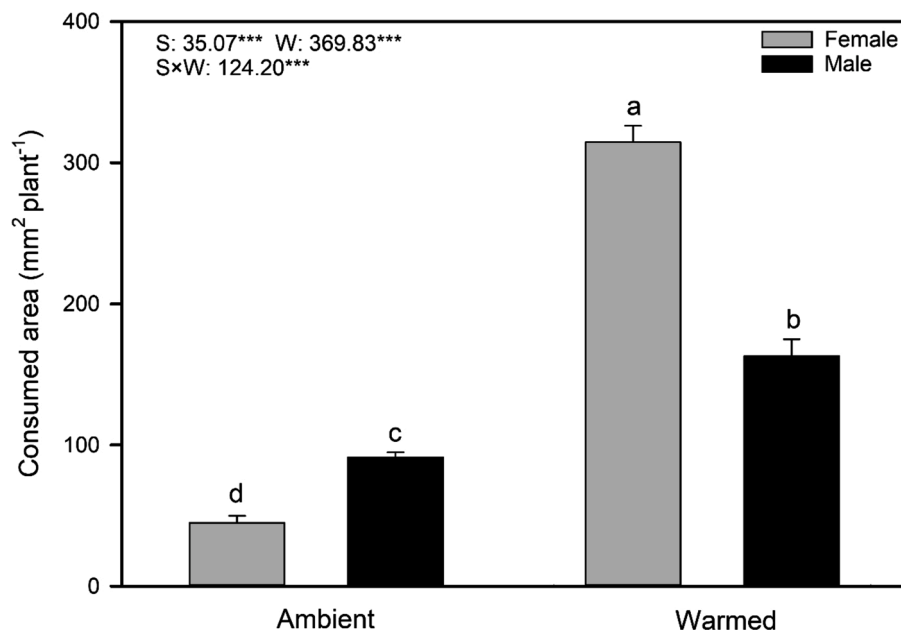


Fig. 2. Consumed area by the leaf-chewing pest *Spodoptera litura* in female (gray bars) and male (black bars) *Populus cathayana* leaves under warming treatments. The same letters above the bars indicate no significant difference among treatments at $P < 0.05$ according to Tukey's HSD test.

toughness and LMA were influenced by the interaction of sex and warming treatment.

3.4. Sexual differences in leaf secondary metabolites under warming

Under non-feeding conditions, leaf concentrations of salicortin were not influenced by sex or warming treatment, yet condensed tannins CT concentrations were influenced by the warming treatment (Table 1). Female and male salicortin concentrations were similar among treatments under each temperature condition (Fig. 4a). CT concentrations were similar between sexes under each temperature condition, but in females, the concentrations of CT increased under while male concentrations did not change significantly (Fig. 4b).

Spodoptera litura feeding caused increased leaf concentrations of salicortin and CT, the strength of which was related to both sex and warming (Table 1; Fig. 4c–f). Post-feeding and feeding responses of female salicortin concentrations were higher than those of males under ambient condition, but they were lower than for males under warmed condition (Fig. 4c, e). Warming treatments decreased the concentration and response of female salicortin but we did not see this same decrease in males. For CT, post-feeding and feeding responses of male and female individuals were similar under ambient conditions, but female CT responses were lower than for males under warmed condition (Fig. 4d, e). Warming treatment did not influence female or male post-feeding CT concentrations, while the responses of both sexes decreased, but especially in females (Fig. 4d, e). We find support for the interaction of sex and warming treatment in the feeding responses of salicortin and CT.

3.5. Sexual differences in leaf activities of defense enzymes under warming

Activities of polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL) and lipoxygenase (LOX) were affected by warming and plant sex, while superoxide dismutases (SOD) and peroxidase (POD) were affected by warming but not by sex (Table 2). Most of these traits were also affected by the interaction of sex and warming treatments. For plants not subject to *Spodoptera* feeding, warming increased the activity of SOD, female POD and PPO and male PAL, while male POD, PPO, LOX and female PAL activities were similar between ambient and warming

treatments. Under ambient temperatures, females had lower SOD and POD, similar PPO and LOX and higher PAL activities than in males, while females had higher SOD and POD, similar PAL and lower PPO and LOX activities than in males under warming condition.

S. litura feeding caused increases in all these enzyme activities, the strength of which was related to both sex and warming (Table 2). Overall, we found that warming weakened the feeding response of leaf enzymes to herbivory, especially in females. The herbivore-induced responses of SOD and POD from warming-treatment plants increased in females but were not effected in males. Herbivore-induced activities of PPO, PAL and LOX decreased in females treated by warming, but these traits (except LOX) increased in males. Herbivores induced lower SOD and higher PPO, PAL and LOX activities in females than in males growing under ambient temperature conditions, while lower PPO and LOX were observed in warming-treatment females than in males. Moreover, feeding-induced responses in SOD, POD, PPO, PAL, LOX activities between the two sexes were similar under the warming treatment, but were higher for POD, PPO and LOX and lower for PAL in females than males under ambient conditions (Table 2).

3.6. Sexual differences in leaf concentrations of salicylic acid (SA) and jasmonic acid (JA) under warming

Leaf SA and JA concentrations in the post-feeding plants and their feeding responses were significantly influenced by the warming treatment. JA was also influenced by sex and by the interaction of sex and warming treatment, but this was evident only in the feeding trial plants (Table 3). Warming decreased JA contents in both sexes in the non-feeding or post-feeding treatment, but did not significantly influence SA (Fig. 5a–d). In all cases, warming decreased the SA and JA responses to feeding in each sex (Fig. 5e, f). In non-feeding plants, leaf concentrations of SA and JA were similar between both sexes under ambient or warmed condition, while female JA was increased and SA remained similar to levels under ambient conditions (Fig. 5a–d). In addition, the feeding-induced response of female JA was higher than males under ambient temperatures, but was lower under warming conditions (Fig. 5f).

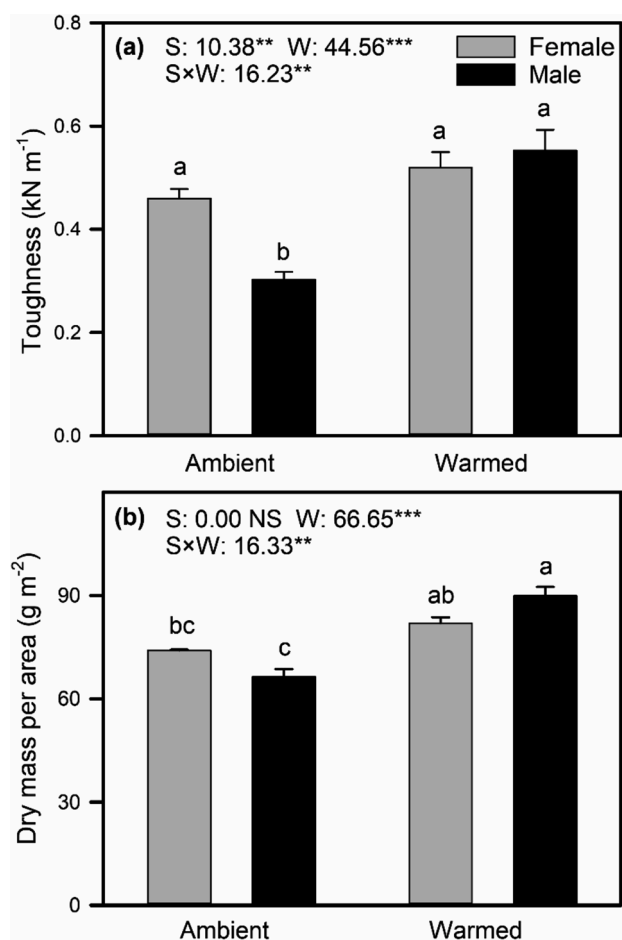


Fig. 3. Toughness (a) and dry mass per area (b) of female (gray bars) and male (black bars) *Populus cathayana* leaves under warming treatments. The same letters above the bars indicate no significant difference among treatments at $P < 0.05$ according to Tukey's HSD test. S, sex effect; W, warming effect; S \times W, interaction of Sex and Warming. F-value and P-value are shown. NS, $P > 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 1

Two-way analysis of variance (ANOVA) of the effects of sex, warming and their interaction on the concentrations of salicortin and condensed tannins of *Populus cathayana* leaves.

Traits	Sex		Warming		Sex \times Warming	
	F	P	F	P	F	P
<i>Non-feeding</i>						
Salicortin (mg g^{-1})	0.143	NS	0.03	NS	0.00	NS
Condensed tannins (mg g^{-1})	0.029	NS	13.06	**	0.45	NS
<i>Post-feeding</i>						
Salicortin (mg g^{-1})	2.39	NS	51.02	***	33.38	***
Condensed tannins (mg g^{-1})	7.27	*	6.56	*	1.55	NS
<i>Feeding responses</i>						
Salicortin (%)	0.78	NS	68.06	***	29.68	***
Condensed tannins (%)	6.17	*	178.11	***	8.12	*

NS, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P \leq 0.001$.

3.7. Relationships among consumed leaf area and inducible responses in leaf secondary metabolites and phytohormones

The leaf area consumed by *S. litura* was significantly negatively related to pre-feeding levels of salicortin, condensed tannins (CT), salicylic acid (SA) and jasmonic acid (JA) contents in females and salicortin

and JA contents in males (Fig. 6a–d). Moreover, the consumed area was significantly negatively related to herbivore-induced responses of leaf salicortin, CT, SA and JA in each sex (Fig. 6e–h). Female plants demonstrated steeper relationships between consumed leaf area and these defensive traits for post-feeding plants. In addition, the consumed area in females was always greater than in males at a given insect herbivore-induced salicortin content, JA content or JA response.

4. Discussion

Using both field surveys and controlled experiments, our study demonstrates that sex-based differences in plant herbivore defenses in *P. cathayana* are modulated by temperature. Furthermore, physical, biochemical and physiological analyses show that warming had a sex-specific effect on leaf toughness, leaf dry mass per area (LMA), lignin, salicortin, condensed tannins (CT), as well as on the activity of defensive enzymes, salicylic acid (SA) and jasmonic acid (JA). Warming treatments either resulted in no change or even slightly increased many of these constitutive defensive traits, while biochemical and physiological induced defenses (e.g., salicortin, CT, activities of defense enzymes, SA and JA) decreased under warming. Female defenses including toughness, insect herbivore-induced salicortin, peroxidase (POD), polyphenol oxidase (PPO), lipoxigenase (LOX) and JA generally higher than in males under ambient temperatures, while we observed the opposite trend under warmed conditions. This reversal was mainly caused by increased suppression of induced-defensive traits in females under warming rather than constitutive defense. Our study provides novel insight into how anti-herbivore defenses of dioecious trees respond to elevated temperatures, inverting the typical pattern where females are better defended.

4.1. Effect of elevated temperature on herbivory and constitutive and inducible defenses

Our results show that both the proportion of *P. cathayana* leaves attacked and the average consumed area per attacked leaf were greater at lower elevations, which was similar to previous findings in other tree species (Rasmann et al., 2014; Moreira et al., 2018). Temperature co-varies with elevation, and likely contributes to observed trends in herbivory. In the warming experiment, which controlled insect diversity and abundance, we found higher herbivory under warmed conditions, a similar pattern to the field survey results. Collectively, these results confirm that environmental warming can increase insect herbivory in trees, which has been expected related to reductions in plant defenses against insect herbivory (Zvereva and Kozlov, 2006; Jamieson et al., 2015).

Constitutive and inducible defenses are the main plant strategies against herbivores (War et al., 2012; Karban, 2020). In this study, we found that in the absence of herbivory warming did not significantly decrease the main physical foliar defensive traits (toughness, LMA; Fig. 3) and water content (Fig. S2), while salicortin, CT and SA, male foliar toughness, LMA and female condensed tannins increased under warming. These results suggest that temperature-driven changes in constitutive physical leaf defenses and chemical secondary metabolites could not explain higher levels of herbivory on *P. cathayana* leaves under warming. A recent study in trembling aspen (*Populus tremuloides*) found that different sets of salicinoid phenolic glycosides play roles in plant defense (Cope et al., 2019). In *Populus*, salicortin is thought to be among the most highly abundant salicinoid phenolic glycosides within plant tissues (Massad et al., 2014; Cope et al., 2019; Fabisch et al., 2019). The other phenolic glycosides, including salicin and tremulacin, may be more responsive to different types of insect herbivory (e.g., Fabisch et al., 2019) and abiotic factors (Eisenring et al., 2021). While we have examined salicortin as a primary defensive compound, these secondary metabolites may respond uniquely to environmental drivers and build plant defenses that are complementary to one another. Therefore, it

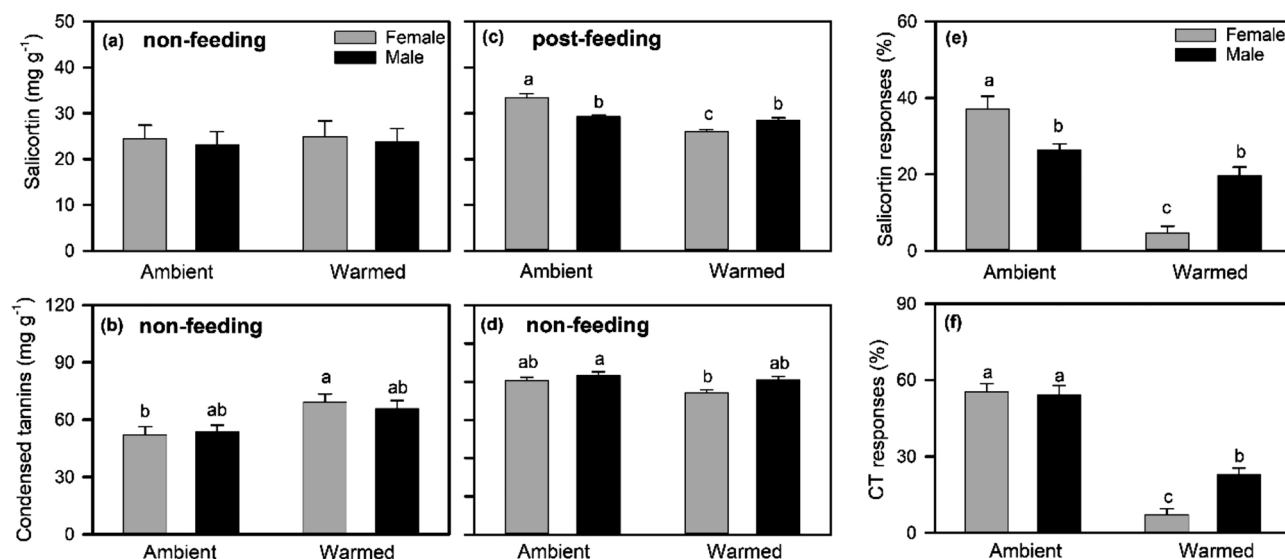


Fig. 4. Concentrations of salicortin and condensed tannins of female (gray bars) and male (black bars) *Populus cathayana* leaves under warming treatments with non-feeding (a, b), post-feeding (c, d) and their responses (e, f). The same letters above the bars indicate no significant difference among treatments at $P < 0.05$ according to Tukey's HSD test. The results of statistical analyses among treatments are given in Table 1.

Table 2

Two-way analysis of variance (ANOVA) of the effects of sex, warming and their interaction on the defensive enzyme activity of *Populus cathayana* leaves.

Traits	Ambient		Warmed		Sex	Warming	Sex × Warming			
	Female	Male	Female	Male			F	P	F	P
Non-feeding										
SOD (U mg ⁻¹ protein)	36.65 ± 1.77d	53.38 ± 2.14c	80.36 ± 2.35a	69.6 ± 1.15b	2.46	NS	247.32	***	52.04	***
POD (U mg ⁻¹ protein)	547.81 ± 60.07c	887.95 ± 49.21b	1543.05 ± 87.97a	1102.91 ± 46.88b	0.63	NS	91.72	***	38.13	***
PAL (U mg ⁻¹ protein)	584.97 ± 48.4a	209.58 ± 45.81b	561.94 ± 41.41a	524.44 ± 28.6a	24.45	***	12.21	**	16.37	**
PPO (U mg ⁻¹ protein)	152.7 ± 13.34b	175.7 ± 11.69b	202.52 ± 13.92b	277.03 ± 13a	14.04	**	33.73	***	3.92	NS
LOX (U mg ⁻¹ protein)	233.57 ± 13.62a	220.91 ± 9.16a	175.62 ± 9.55b	231.54 ± 7.78a	4.45	*	5.32	*	11.17	**
Post-feeding										
SOD (U mg ⁻¹ protein)	60.3 ± 3.04c	79.4 ± 3.28b	89.66 ± 1.5a	79.14 ± 0.64b	3.25	NS	37.43	***	38.78	***
POD (U mg ⁻¹ protein)	1190.29 ± 100.87b	1488.31 ± 46.41ab	1591.48 ± 80.26a	1489.88 ± 53.7ab	1.78	NS	7.49	*	7.38	*
PAL (U mg ⁻¹ protein)	1050.16 ± 45.72a	485.25 ± 21.46c	647.5 ± 23.1b	675.46 ± 11.39b	89.72	***	14.04	**	109.38	***
PPO (U mg ⁻¹ protein)	371.77 ± 16.33a	292.23 ± 10.53b	316.37 ± 5.16b	396.61 ± 5.53a	0.00	NS	5.52	*	58.73	***
LOX (U mg ⁻¹ protein)	636.17 ± 34.83a	489.15 ± 7.72b	292.78 ± 19.8c	433.22 ± 14.45b	0.02	NS	85.12	***	44.11	***
Feeding responses										
SOD (%)	64.53 ± 8.29a	48.73 ± 6.14a	11.58 ± 1.87b	13.7 ± 0.91b	1.69	NS	69.91	***	2.90	NS
POD (%)	117.28 ± 18.41a	67.61 ± 5.23b	3.14 ± 5.2c	35.08 ± 4.87bc	0.75	NS	51.68	***	15.97	**
PAL (%)	79.52 ± 7.81b	131.53 ± 10.24a	15.23 ± 4.11c	28.8 ± 2.17c	22.93	***	148.77	***	7.88	*
PPO (%)	143.46 ± 10.69a	66.32 ± 5.99b	56.22 ± 2.55b	43.16 ± 1.99b	50.61	***	75.49	***	25.55	***
LOX (%)	172.74 ± 14.93a	121.42 ± 3.5b	66.71 ± 11.27c	87.11 ± 6.24bc	2.38	NS	48.91	***	12.81	**

SOD, superoxide dismutases; POD, peroxidase; PAL, phenylalanine ammonia-lyase; PPO, polyphenol oxidase; LOX, lipoxygenase. The same letters in same row indicate no significant difference among treatments according to Tukey's HSD test ($P < 0.05$). NS, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

remains necessary to extend our results to a broader community of attacking insect herbivores and measure a broader suite of chemical defense compounds. This will allow us to understand how these compounds may trade off under warming and buffer plant defenses under changing abiotic conditions that may favor certain defense strategies over others.

Some studies have found that warming decreases leaf toughness of some temperate tree species, and that secondary phenolic metabolites tend to decrease with increasing temperatures (e.g., Zvereva and Kozlov, 2006; Jamieson et al., 2015), which may be affected by primary metabolism and growth (Rasmann et al., 2014). Decreased growth rates under +4 °C warming in this study suggest that the warming treatment suppressed the growth of *P. cathayana* (Fig. S1), possibly due to heat stress (Liu et al., 2020). Increases in leaf toughness as well as LMA, which can be an important adaptive structural response for growing in hot, drought-prone habitats, may provide resistance to abiotic stress

while also decreasing leaf palatability (War et al., 2012). In addition, we found that warming tended to increase the activity of the enzymes SOD, POD, and PPO in non-feeding leaves (Table 2). Higher activity in these defensive enzymes, besides defending plants against insect herbivory, is usually beneficial for defending against oxidative stress, which is detrimental to plants physiologically and may be adaptive to abiotic environmental stress (Zhang et al., 2012). Overall, we found that warming in without herbivory increased physical defense traits and carbon-based secondary metabolites.

Beyond pre-feeding defensive traits, herbivore-induced physiological responses may often be crucial for defending against insect attacks (Fabisch et al., 2019; Karban, 2020). Typically, herbivore attacks activate plant defense genes and trigger defensive enzyme activity in local and systemic tissues (Eyles et al., 2010; Kerchev et al., 2012). An interesting result found from the field survey is that the relationship between elevation and the proportion of attacked leaves (Fig. 1a) was

Table 3

Two-way analysis of variance (ANOVA) of the effects of sex, warming and their interaction on concentrations of salicylic acid and Jasmonic acid of *Populus cathayana* leaves.

Traits	Sex		Warming		Sex × Warming	
	F	P	F	P	F	P
<i>Non-feeding</i>						
Salicylic acid (ng g ⁻¹)	2.04	NS	0.79	NS	0.21	NS
Jasmonic acid (ng g ⁻¹)	0.01	NS	12.35	**	0.01	NS
<i>Post-feeding</i>						
Salicylic acid (ng g ⁻¹)	2.96	NS	13.23	**	0.01	NS
Jasmonic acid (ng g ⁻¹)	16.20	**	286.99	***	66.38	***
<i>Feeding responses</i>						
Salicylic acid (%)	3.32	NS	34.91	***	2.47	NS
Jasmonic acid (%)	6.13	*	215.84	***	51.63	***

NS, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

less steep than for the consumed leaf area (Fig. 1b), which suggests that responses of induced defenses may be more important than constitutive defenses in driving overall responses to temperature. We further investigated herbivory-induced salicortin, CT, defensive enzyme activity (SOD, POD, PAL, PPO, lipoxygenase (LOX)), SA and JA in feeding trials with the generalist herbivore, *Spodoptera litura*, from ambient or warming-treatment plants, and found that herbivore-induced response traits decreased under warming. Additionally, higher levels of these carbon-based secondary metabolites and phytohormones reduced the area of leaf consumed (Fig. 6). These results indicate that warming reduced the inducibility of chemical defenses in *Populus cathayana* seedling, which generally aligns with finding in oak trees (Galván et al., 2018). Thus, weakened induced defense may explain higher leaf herbivory under warming.

Overall, our results indicate that lowered defenses against insects in *P. cathayana* due to warming are mainly driven by changes in induced defenses rather than constitutive defenses, which may be related with a warming-mediated shift in metabolic processes underlying the allocation of carbon to structural versus defense compounds (Jamieson et al., 2015). Compared to abiotic stress (e.g., climatic, nutrient), insect herbivory damage is more sporadic and occurs over a shorter time period. Under ambient conditions, *P. cathayana* showed high induced defense rather than constitutive defenses, while high temperatures led to

decreased induced defense and maintained constitutive defenses, which may be an important part of ecological and evolutionary strategies to deal with carbon allocation tradeoffs for dealing with stress in plants (Moreira et al., 2014; Rasmann et al., 2015; Züst and Agrawal, 2016). Although these defense strategies could also contribute to heat stress resistance, they could decrease the efficiency of defending against biotic stress, as induced defense has been viewed as a more cost-efficient strategy and as more effective against insect herbivory than constitutive defenses in some circumstances (Karban, 2020).

4.2. Sexual dimorphism in defense in conjunction with temperature change

Our data from field surveys and the controlled experiment suggest that defense against insect herbivory depends on temperature differently for males and females. Sex-specific defense in *Populus* is environmentally context-dependent (Liu et al., 2021; Randriamanana et al., 2014; Zhang et al., 2019). Under ambient conditions, females experienced lower consumption of leaves than males, such that females performed better against herbivory than males, a result which is similar to studies of other woody species (Cornelissen and Stiling, 2005). However, their salicortin and CT concentrations remained similar between the two sexes (Figs. 1 and 4; Fig. S1). On the other hand, we found that female leaves had a greater toughness than male leaves, which should be advantageous against chewing herbivores. Moreover, it has been generally assumed that females invest more into reproduction and defense compared to male plants, and thus might exhibit lower vegetative growth (Cornelissen and Stiling, 2005; Randriamanana et al., 2014). In our study, however, we found that the growth rate of females was higher than that of males under ambient temperatures, which suggests that females did not follow the typical trade-off where they both grow better and are better defended under the ambient temperature, which may be related with carbon allocation through biosynthetic pathway (growth and defense) (Randriamanana et al., 2014) or through defense compounds (e.g., salicinoid phenolic glycosides and condensed tannins) (Cope et al., 2019). Females with high growth rates may produce more plant primary metabolites, such as lignin, decreasing leaf palatability. In addition, the activity of PAL, a crucial factor in the biosynthesis of lignin, also supports this pattern. On the other hand, inducible defenses in females were higher than in males, suggesting that females are more responsive to insect herbivory under ambient conditions. Thus, our study of physical, chemical and physiological traits collectively explain how faster

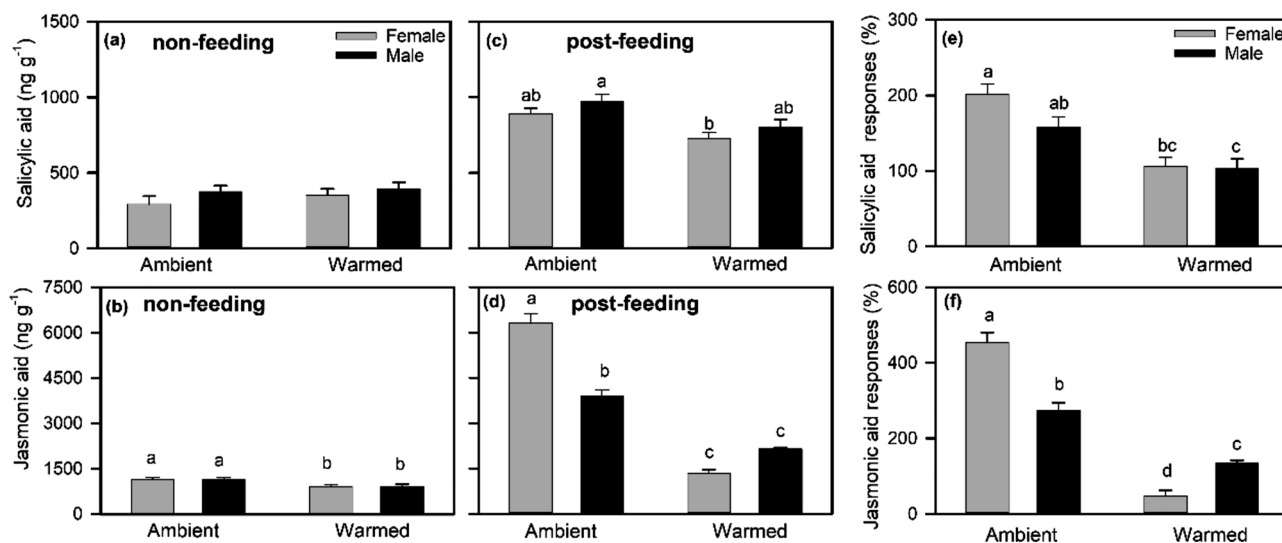


Fig. 5. Levels of salicylic acid and jasmonic acid and of female (gray bars) and male (black bars) *Populus cathayana* leaves under warming treatments with non-feeding (a, b), post-feeding (c, d) and their responses (e, f). The same letters above the bars indicate no significant difference among treatments at $P < 0.05$ according to Tukey's HSD test. The results of statistical analyses among treatments are given in Table 2.

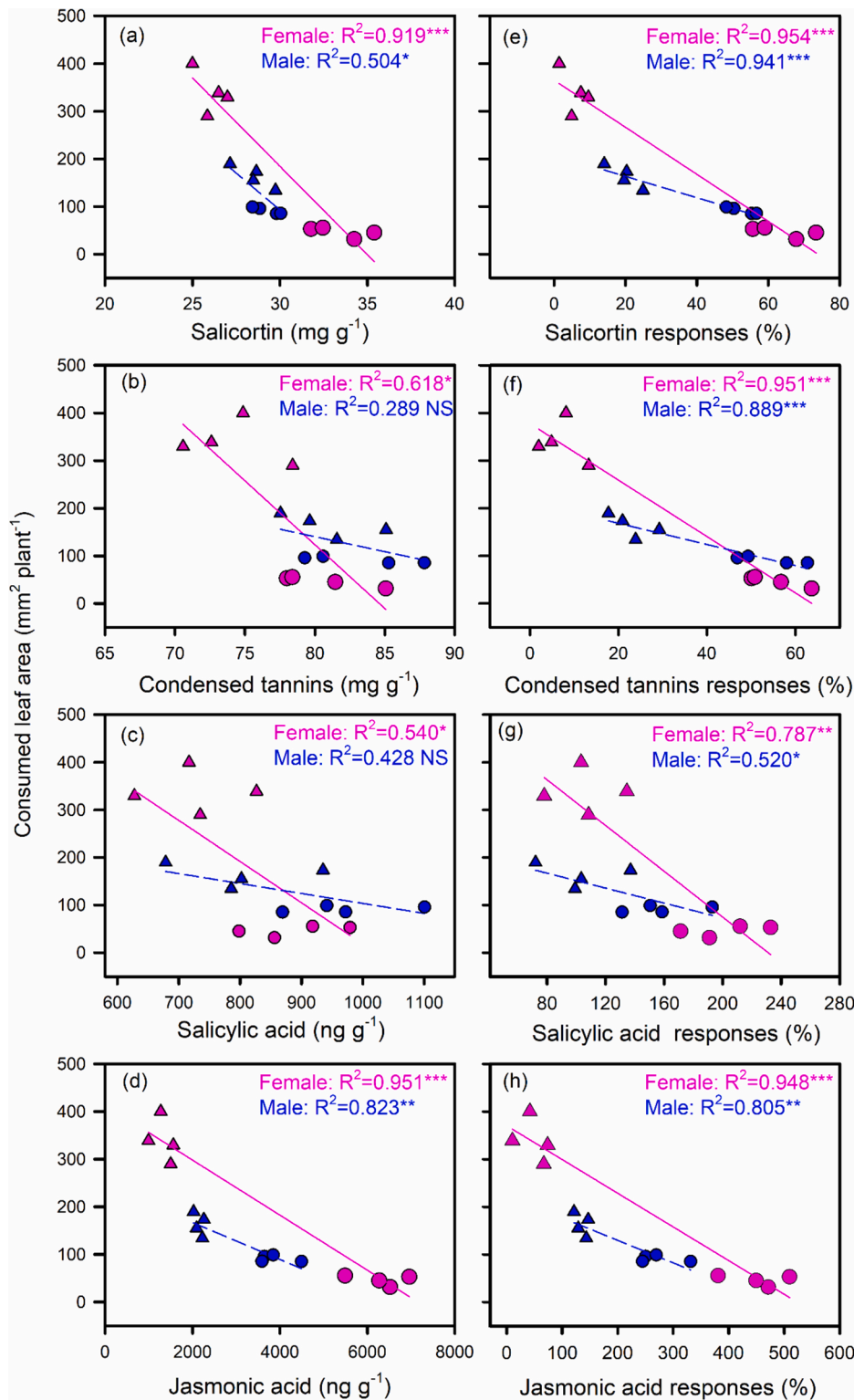


Fig. 6. Relationship between consumed leaf area by the leaf-chewing pest *Spodoptera litura* and leaf concentrations of salicortin, condensed tannins, salicylic acid, jasmonic acid with post-feeding (a-d) and herbivore-induced responses (e-h) across ambient (circle) and warmed (triangle) treatments of female (pink circle and triangle; pink solid line) and male (blue circle and triangle; blue dotted line) *Populus cathayana*. (NS, $P > 0.05$; *, $P < 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

growing females are better defended against insect herbivores, which may be an important ecological strategy in dioecious plants. It may be beneficial for females to maintain higher growth and defense during vegetative periods to compensate for higher costs during reproduction (Barrett and Hough, 2013). It is also thought that male-biased herbivory may act as an important selective pressure during the evolution of separate sexes from hermaphroditism to dioecy via gynodioecy (Ashman, 2002).

Sex-specific biochemical and physiological responses to environmental changes have been found in *Populus*, yet previous studies have mainly focused on resistance to abiotic stress (e.g., Melnikova et al., 2017; Liu et al., 2020). We found that insect herbivory on *P. cathayana* leaves, along with chemical and physiological responses in defense against herbivory were influenced by the interaction of sex and warming, which builds on previous results that poplar defense is influenced by the interaction between genes and the environment (Barker et al., 2019).

Herbivore-induced secondary metabolites and phytohormones are related to reduced leaf area consumption under ambient conditions and higher consumption of females than of males under warmed conditions. Female *P. cathayana* has previously been shown to be higher sensitivity to environmental change than males (Xu et al., 2008; Liu et al., 2020) and similarly here, females respond more in induced defenses under high temperature treatments. Female *P. cathayana* defense was lower than that of males under high temperature which maintained their physiological and chemical defenses. Lower salicortin and JA levels may be respectively caused by reduced PAL and LOX activities, as PAL is a key enzyme involved in the synthesis of phenolic metabolites through the shikimate-phenylpropanoid pathway in *Populus* and LOX is the key enzyme involved in deriving JA from linolenic acid via the octadecanoid pathway (Chen et al., 2009). Although these results tell us a lot about sex-specific defense over a relatively short time period, sexual chemical defensive differences may be part of long-term adaptive defense strategies (Cope et al., 2019).

According to life-history theory, organisms face trade-offs between growth, reproduction, and defense (Barrett and Hough, 2013), but we expect that physiological adjustments required by climate change will mediate resource allocation among these functions (Morales and Munné-Bosch, 2016). The results that we have observed involving sex-biased herbivore responses mediated by temperature suggest that there may be a trade-off between plant reproduction and defense rather than between growth and defense. Besides contributing to defense against insect herbivory, antioxidative defense is also crucial for plant metabolic processes and survival under environment stress (Kerchev et al., 2012; Morales and Munné-Bosch, 2016). Greater increases in SOD and POD activities in females under warming conditions suggest that females suffer from higher oxidative stress; weakened female defenses under warming may compensate for growth reductions due to this stress. The role of oxidative physiological stress in life-history trade-offs would be a fruitful avenue for further research.

Herbivory is an important ecological force influencing plant survival, which may have important consequences in determining sex ratios in natural populations and even sexual niche differentiation in dioecious species. Phytophagous insects, which are abundant in forests, can affect tree growth and survival, and can be the cause of major landscape disturbances (Jamieson et al., 2015). The results of this study suggest that

female *P. cathayana* may be at higher risk of mortality due to weakened defenses under climate warming, which could push *P. cathayana* populations towards male-biased sex ratios in the future. Beyond the ecological importance of *P. cathayana*, our findings may have practical implications for forest management of this economically important forestry species. Long term studies of sex-specific plant defenses under multiple climate drivers (e.g. CO₂, drought), as well as investigations into the responses of other plant tissues (e.g. roots, stems), are needed to understand how our results may apply across the full span of ontogenic development, which would allow for more comprehensive forecasts of forest responses to climate change.

5. Conclusion

Results of this study show, for the first time, that warming suppresses induced defenses in females more than in males, inverting the typical pattern of insect herbivore defense across the sexes (Fig. 7). These results are mainly driven by changes in inducible defense (salicortin, defensive enzyme activities and JA) rather than changes in constitutive defensive traits. These findings highlight sex-specific physical, chemical, and physiological responses to biotic stress that may increase the vulnerability of dioecious plants to shifts in population dynamics under ongoing global warming.

Author contributions

TD and RZ collected the data, TD analyzed the data and wrote the manuscript, JL had a significant contribution to the first manuscript, JCF and TEXM contributed to writing and editing, and XX designed experiment and approved the manuscript; all authors read and revised the final manuscript.

Author statement

The authors found that sex-specific leaf defense against insect herbivory in *Populus cathayana* can be mediated by climate warming, and that higher leaf herbivory in females under warming is mainly influenced by decreases in induced defense responses rather than constitutive defense responses. These findings highlight sex-specific physical,

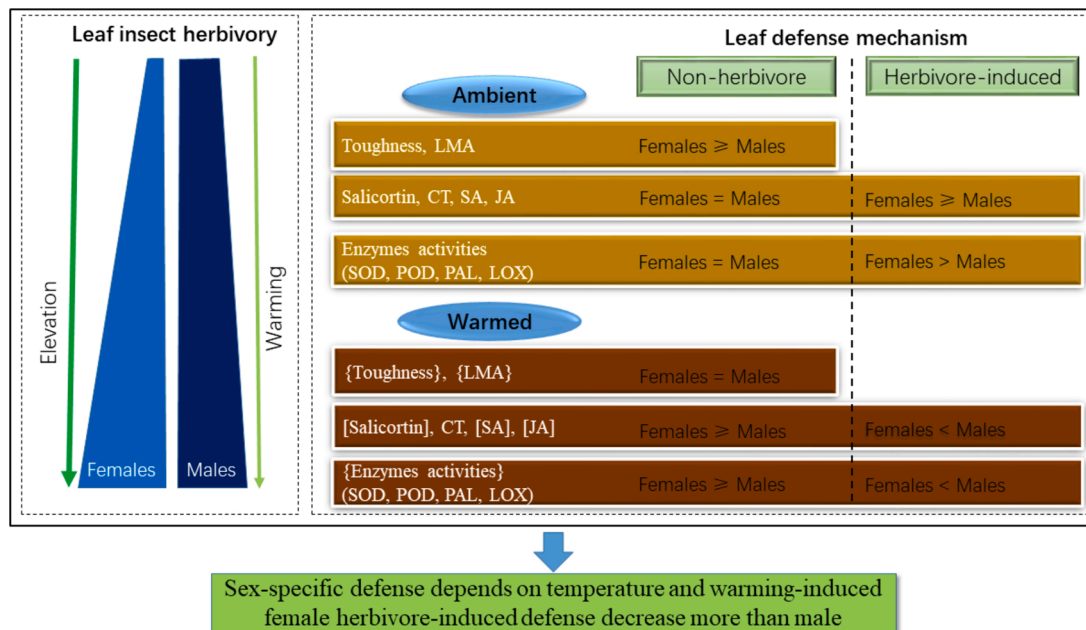


Fig. 7. Schematic diagram of the important findings of sexual-related defense in *Populus cathayana* under warming experiment. Brace on traits in warmed condition mean warming increased the trait; bracket on traits in warmed condition mean warming decreased the trait.

chemical, and physiological responses to biotic stress that may increase the vulnerability of dioecious plants to shifts in population dynamics under ongoing global warming.

This paper is neither the entire paper nor any part of its content has been published or has been accepted elsewhere. It is not being submitted to any other journal. The submission of this paper for publication has been approved by all authors and relevant institutions.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.envexpbot.2021.104557>.

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