Ellagitannins from the Onagraceae Decrease the Performance of Generalist and Specialist Herbivores

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Abstract

Phenolics have a known role in defences against herbivores, but the defensive function of specific groups of phenolics is still poorly understood. For example, ellagitannins (a type of hydrolysable tannin) are predicted to decrease insect herbivore performance, but the effect of different types of ellagitannins on generalist and specialist herbivores has rarely been assessed. Here, we test the effects of the dominant oligomeric ellagitannins of *Oenothera biennis* and other Onagraceae on herbivore performance. We fed artificial diet containing between 1 and 100 mg/g of polyphenol fractions with varying amounts and compositions of dimeric oenothein B, the trimeric oenothein A and larger oligomers, to one generalist (*Spodoptera exigua)* and one specialist (*Schinia florida*) insect herbivore species. We compared the effects of these ellagitannin fractions on herbivore performance to the effects of artificial diet containing total phenolic extracts from *O. biennis*, which contained these ellagitannins as well as many additional phenolic metabolites including flavonoid glycosides and caffeic acid derivatives. Both the ellagitannin fractions and *O. biennis* phenolic extracts had strong negative effects on *S. exigua* and *S. florida* performance, with stronger effects on the generalist herbivore. Differences between the effects of ellagitannin fractions were small and dependent on insect life stage. The defensive effects of these ellagitannins were large, with concentrations as low as 0.1% leading to lethality. These results highlight the important defensive function of ellagitannins against specialist and generalist herbivores and the need to characterize the effects of these understudied phenolics.

Key Words-Chemical ecology, bioassay, oenothein, phenolics, *Spodoptera exigua*, tannins.

Introduction

Plant secondary metabolites play a large role in defending plants against herbivores. Yet, despite the importance of many secondary metabolites in defence (Denno and McClure 1983; Fritz and Simms 1992; Johnson 2011; Rosenthal and Berenbaum 1991; Wallace 1976; Walters 2011), it is often difficult to demonstrate the defensive function of secondary metabolites. For example, a meta-analysis of 72 quantitative genetics studies failed to find a consistent effect of genetic variation in the concentration of secondary metabolites on herbivore performance (Carmona et al. 2011). This study identified important limitations of previous work examining the defensive function of secondary metabolites, such as the difficulty of establishing the effects of secondary metabolites that have diverse classes of compounds (e.g. phenolics, alkaloids, glucosinolates) with many biological functions. Similarly, the toxicity of chemical defences may be due to additional factors that are difficult to quantify in genetically-based tests, such as synergism between compounds and compound diversity. Focusing on the effects and modes of action of a few, structurally similar compounds could yield more accurate tests of the defensive function of secondary metabolites (Carmona et al. 2011; Salminen and Karonen 2011). We take such an approach to understand the defensive function of macrocyclic ellagitannin oligomers, an abundant type of hydrolysable tannin found in many Onagraceae species and other plant species that has been associated with putative defensive function against insect herbivores (Agrawal et al. 2012; Johnson et al. 2009).

Tannins are among the most widespread group of secondary compounds and are ubiquitous in plants. Tannins are broadly divided into condensed tannins (proanthocyanidins) and hydrolysable tannins, which include both simple gallic acid derivatives, gallotannins, and ellagitannins (Niemetz and Gross 2005; Quideau and Feldman 1996; Salminen and Karonen

2011). Many of these compounds are recognized for their role in plant defences against insect herbivores and pathogens (Harborne and Williams 2000; Salminen and Karonen 2011). The defensive function of tannins may be associated with the ability to bind proteins in the guts of herbivores, thereby limiting nutrient uptake (Feeny 1976). With insect herbivores, especially lepidopterans with alkaline guts, tannins more likely cause oxidative stress in the insect gut (Appel 1993; Salminen and Karonen 2011), including oxidative damage to nutrients (Felton and Summers 1995). Additionally, ellagitannins might act as an anti-feedant in which their presence makes the food unpalatable. Here we focus on the defensive role of ellagitannins, which are the dominant group of tannins found in many plant families (Johnson et al. 2014; Moilanen et al. 2015; Salminen et al. 2001; Salminen et al. 2002; Salminen et al. 2004; Yarnes et al. 2006).

Recent work has shown that ellagitannins may be the most bioactive class of tannins, although different ellagitannin compounds exhibit six-fold variation in both oxidative activity (Moilanen and Salminen 2008), as well as in protein precipitation capacity (Karonen et al. 2015). The effect of ellagitannins on insect herbivores has been confirmed by feeding herbivores low to medium-level concentrations (15 mg/g) of ellagitannin mixtures (pedunculagin and pentagalloyl glucose) (Barbehenn et al. 2009), and by placing high concentrations of individual ellagitannins (50 mg/g of vescalagin) in artificial insect diet fed to generalist and specialist herbivores (Roslin and Salminen 2008). These analyses experimentally showed the potential importance of ellagitannins in defence, but they did not compare varying levels of the major compounds present in plant chemical defence profiles.

Field experiments have shown considerable variation in the concentrations of different macrocyclic ellagitannin oligomers within and between species of the Onagraceae (Anstett et al. 2015; Johnson et al. 2009; Parker et al. 2012; Baert et al. 2017). For example, there is large

heritable variation within *Oenothera biennis* for the concentrations of the ellagitannin compounds oenothein A (trimer of tellimagrandin I) and its precursor oenothein B (dimer of tellimagrandin I) (Anstett et al. 2015; Johnson et al. 2009; Parker et al. 2012). Larger oligomers (with up to eleven subunits) also occur within *O. biennis* (Karonen et al. 2010; Salminen et al. 2011) and in other species of the Onagraceae such as *Epilobium angustifolium* (Baert et al. 2015, 2017). The variation in the concentrations of macrocyclic oligomers has been shown to impact insect herbivore performance (Agrawal et al. 2012; Anstett et al. 2015). Specifically, higher levels of oenothein A were negatively genetically correlated with damage by specialist flower and fruit herbivores across 137 genotypes of *O. biennis*, while its precursor oenothein B was positively genetically correlated with increased herbivory (Anstett et al. 2015). *Oenothera biennis* also evolved greater levels of oenothein A in response to selection by specialist seed predators (Agrawal et al. 2012). While a negative effect of oenothein A on herbivores was implicated by these studies, these results were correlative and a direct experimental confirmation of the defensive function of these ellagitannins is still needed.

Here we experimentally test the defensive function of the most abundant ellagitannins in *O. biennis* on generalist and specialist insect herbivore species. We compare the defensive role of macrocyclic ellagitannin oligomers to the effects of the total composition of phenolics from *O. biennis*, which includes flavonoids, caffeic acid derivatives and ellagitannins. We compare the defensive function of ellagitannins to total phenolic extracts by experimentally varying the concentrations of these phenolic compounds within artificial diet fed to one generalist insect herbivore species (*Spodoptera exigua*), and one specialist insect herbivore species (*Schinia florida*). Using a series of these feeding trial experiments, we ask: (1) Do macrocyclic oligomeric ellagitannins decrease the performance of generalist and specialist insect herbivores? (2) How do

fractions with varying amounts of oenothein B, oenothein A, and larger oenothein oligomers, differ in their effects on herbivores? (3) How do the antiherbivore effects of these ellagitannins compare to the effects of the total mixture of phenolic compounds present in *O. biennis?* Our results provide direct experimental evidence that macrocyclic ellagitannin oligomers are a potent chemical defence against generalist and specialist insect herbivores of *O. biennis*.

Methods and Materials

Study System *Oenothera biennis* L. (common evening primrose, Onagreaceae) is a biennial herbaceous plant that grows in open habitats throughout eastern North America. This species is consumed by a diverse community of insect herbivores (Johnson and Agrawal 2005; Johnson and Agrawal 2007), and contains a diversity of defensive chemicals including a large concentration of ellagitannins (>5% dry weight) (Agrawal et al. 2012; Anstett et al. 2015; Johnson et al. 2009). *Oenothera biennis* also experiences a genetically based trade-off between its two most abundant ellagitannins, oenothein B and oenothein A (Anstett et al. 2015; Johnson et al. 2009). Oenothein B and A are typically the dominant ellagitannins in *Oenothera spp*, whereby oenothein B contains two subunits of tellimagrandin I and oenothein A contains three subunits (Fig. 1). These compounds contain a large number of phenolic rings with oxidatively important groups (such as hexahydroxydiphenoyl groups) that can cause oxidative stress and damage to nutrients in the alkaline guts of insects (Appel 1993; Barbehenn et al. 2006; Salminen and Karonen 2011; Salminen et al. 2011).

We used larvae of the herbivorous moth *Spodoptera exigua* (Noctuidae: Lepidoptera) as a model to study the defensive effects of phenolics and ellagitannins on a generalist caterpillar. *Spodoptera exigua* is frequently used in laboratory experiments because of its extremely

generalized diet, which includes >37 plant families (Normark and Johnson 2011). *Spodoptera exigua* eggs were acquired from Benzon Research (Carlisle, PA, USA), and were reared on a soy-flour and wheat germ-based diet (Beet Armyworm Diet, Sutherland Products, Mayodan, NC, USA). The insects were maintained in a colony with >100 mating individuals for 2-3 generations before being utilized in experiments.

Schinia florida (Noctuidae: Lepidoptera) larvae have a specialized diet that includes the flowers and fruits of *O. biennis* and close relatives (Sargent 1969). High levels of ellagitannins and in particular oenothein A are correlated with decreased damage to *O. biennis* (Agrawal et al. 2012; Anstett et al. 2015), making this moth a useful model system to test the direct effects of ellagitannins on specialist insect herbivores. Second and third instar *Schinia florida* were collected from the field in August 2016 in Mississauga and King City, ON, Canada, and used directly in feeding experiments. Our attempts to have earlier instars feed on artificial diet were unsuccessful and previous attempts to rear colonies of *S. florida* have never been successful (A. Agrawal Pers. Comm.). We were successful at getting *S. florida* to feed on Beet Army Worm diet without any apparent aversion starting at the 2nd instar, and these larvae were able to successfully complete development until pupation.

Experimental Design To test for the effect of phenolics and the differences in the defensive function of ellagitannin compounds, we selected two genotypes known to have high levels of oenothein B and low concentrations of oenothein A, and two genotypes with high concentrations of oenothein A and low concentrations of oenothein B (Table S1). These genotypes also contained a diversity of ~70 other phenolic compounds, which included flavonoid glycosides and caffeic acid derivatives (Salminen unpublished data). Seeds were germinated on moistened filter paper within sealed petri dishes exposed to a full spectrum of sunlight. Forty seedlings of each

genotype were grown for six months in a growth chamber (Conviron CMP6050, Winnipeg, Canada). The chamber was set to a 16:8 h day:night cycle with a 25 °C day: 20 °C night temperature regime, a ramp rate of 1 °C/h, and 500 μmol/m^2/s of light intensity during the day. Fully expanded, non-senescing leaves were collected and stored in a -80° C freezer before phenolic extraction. Previous work on *Oenothera biennis* found that the constitutive concentration of total phenolics grown in a controlled environment varied between 45 and 160 mg/g (Anstett et al. 2016) depending on the genotype, which falls within the range of the concentration of total phenolics observed from plants growing in the field (Anstett et al. 2015).

We purified individual fractions of oenothein oligomers, excluding other types of phenolics and many classes of ellagitannins to specifically test the effects of oenothein B, oenothein A, and higher oligomers from the confamilial plant species *Epilobium angustifolium* (Onagraceae) as outlined in Baert et al. (2015). Importantly, macrocyclic tellimagrandin I –based ellagitannins are identical and interchangeable between *E. angustifolium* and *O. biennis* (Baert et al. 2015; Baert et al. 2016; Karonen et al. 2010; Salminen et al. 2011). Briefly, these fractions were obtained by maceration of *E. angustifolium* flower tissue in an 80% aqueous acetone solution for 48 h followed by filtering and lyophilizing. This procedure was carried out many times yielding hundreds of grams of extract. A fractionation step was then carried out by putting a mixed slurry containing flower extract through four elutions of water (fraction I), methanol/water (1:1, v:v, fraction II), methanol (fraction III), and finally, acetone/water (4:1, v:v, fraction IV), using Sephadex LH-20 gel in a Büchner funnel. Methanol and acetone was then evaporated and the remaining solutions were lyophilized again. The final fractions used in the experiments contained only ellagitannins and no other phenolics. Fraction II was enriched for

oenothein B, whereas fraction IV was enriched for oenothein A plus larger oligomers of tellimagrandin I (Table S2).

Insect diet was made to contain varying concentrations of either ellagitannin fraction II, ellagitannin fraction IV, or the total phenolic extracts. The ellagitannin fraction diets contained 0, 1.0, 2.5, 5.0, 10.0, 50.0, and 100.0 mg/g (dry weight) of either fraction II or IV (i.e., 0 to 10% of ellagitannins per unit mass of diet). The total phenolic extract diets contained a concentration of 0, 1.0, 2.5, 5.0 or 10.0 mg/g (dry weight) of total phenolics (i.e., 0 to 1% of phenolics per unit mass of diet). Greater phenolic concentrations were not included because concentrations >10 mg/g resulted in 100% mortality for the generalist caterpillar. Total phenolics extracts, as well as ellagitannin fractions, were applied to the food mixture suspended in ddH_2O (Milli-Q Reference System, Millipore). Approximately 4 mL of food was made for each insect every five days by mixing insect food with boiling water at a ratio of 0.7 g to 3 mL. After heating and stirring for 2 min, 1 mL of water containing the appropriate amount of total phenolic extract or ellagitannin was added. Controls only received water. This mixture was heated and stirred for 1.5 minutes and then transferred to small plastic cups. These cups were left to cool and set prior to commencing the feeding assay. We analyzed the diet containing phenolics and ellagitannins using ultraperformance liquid chromatography connected to diode array and mass spectrometry detectors (UPLC-DAD-MS/MS), which confirmed that our diet preparation did not affect the diversity and concentration of phenolics in the food (data not shown).

Two feeding experiments were conducted to study the effects of phenolics and ellagitannins on *S. exigua*. One experiment assessed the effects of total phenolic extracts on herbivore performance, and the other assessed the effects of ellagitannin fractions on herbivore performance. In each experiment, fresh food was prepared for every live caterpillar on days 0, 5,

10 and 15. Measurements were made across these same time intervals to allow for an equivalent comparison and high replication among both herbivore species and across the different treatments. For each replicate, one freshly hatched caterpillar was transferred to each food container on day 0 and the caterpillar was subsequently allowed to feed and develop. To assess the effects of phenolics on the generalist caterpillar's performance, we had five replicate caterpillars per phenolic concentration (1.0, 2.5, 5.0, and 10.0 mg/g), for each of the four *O. biennis* plant genotypes. Additionally, we had 32 control generalist caterpillars (for the 0 mg/g levels of total phenolics) (see Table S3 for degrees of freedom in each analysis). The second experiment investigated the effects of ellagitannin fractions. For each fraction we had 10 replicate caterpillars per concentration (1.0, 2.5, 5.0, 10.0, 50.0, 100.0 mg/g) and 15 generalist caterpillars received the control diets (0 mg/g) (see Table S4 for degrees of freedom in each analysis).

Similar experiments were performed on the specialist *S. florida* caterpillars. Food was prepared every 5 days, and we prepared additional food on day 20 to accommodate the longer developmental time of the specialist caterpillars. For the feeding assays that used total phenolic extracts, we had three replicate caterpillars for each of two genotype treatments (see Table S5 for degrees of freedom in each analysis). For the feeding assay that used ellagitannin fractions in the diet, we had five replicate caterpillars for each concentration (1, 5, 10, and 50 mg/g) of both ellagitannin fractions, plus five control caterpillars (0 mg/g) (see Table S6 for degrees of freedom in each analysis). This lower replication compared to the experiments involving *S. exigua* was due to the practical constraints of obtaining a sufficient number of wild caught *S. florida* caterpillars for experiments.

In all experiments, we collected data on live caterpillar mass and survival throughout each experiment. Starting on day 5, caterpillars were weighed every 5 days to determine the effects of the treatments on caterpillar growth. Specialist caterpillars were also weighed at day zero because they varied in initial size; generalist caterpillars were placed on dishes immediately after hatching from eggs and were too small to weigh. Caterpillar survival was tracked for every caterpillar until pupation or death.

Statistical Analysis We used analysis of variance and analysis of covariance to test how the concentration of phenolics, *O. biennis* genotype, and ellagitannin fraction affected the performance of the generalist and specialist herbivores. Caterpillar mass was log (x+1) transformed to improve the fit of the residuals from statistical models to the assumptions of normality and homogeneity of variance. Similarly, the concentration of phenolics and ellagitannin fractions were also log (x+1) transformed. Initial mass was used as a covariate for all analyses involving the specialist caterpillar. First, we carried out comparisons between controls and mid-level phenolic concentrations (5 and 10 mg/g treatments), and between controls and mid-level oenothein concentrations (5 and 10 mg/g). These data were analyzed using the *Anova* command in the Car package of R (R Core Development Team 2018). The objective of this analysis was to give a general assessment of the effects of medium to high concentrations of chemical defences on herbivore performance. Next, we tested how insect mass at each individual time period and mortality were affected by treatment group (genotype or fraction) and concentration of phenolics. These analyses were also carried out using the *Anova* command in the Car package of R.

Results

Generalist Herbivore Performance, Total Phenolic Extracts *Spodoptera exigua* caterpillars fed control diets (0 mg/g) strongly outperformed caterpillars that consumed diets containing any amount of total phenolics (Fig. 2A, Table S3). Control caterpillars had substantially greater mass ($P < 0.001$, Fig. S1A), and greater survival ($P < 0.001$, Fig. 3A), than those fed 5 to 10 mg/g of phenolics (Table S3). Even the lowest concentrations of total phenolic extracts (1 mg/g, 0.1% total phenolics) caused a dramatic decrease in insect mass, compared to the control diet (Fig. 2A). Increased concentrations of phenolics did not lead to further reductions in caterpillar performance (Fig. 2A, Table S3), indicating that total phenolics had a clear negative effect on caterpillar performance. For example, while caterpillar survival was nearly 100% on control diets, no caterpillars survived to pupation when feeding on diet containing any concentration of total phenolics (Fig. 3A). Plant genotypes varied in their effects on the mass of caterpillars (Fig. S2A,Table S3). Genotype and total phenolic concentration also interacted to affect caterpillar mass (Table S3). Caterpillars showed lower mass with increased total phenolic concentrations for all genotypes except genotype 751 (high oenothein B), which was associated with increased performance of caterpillars at higher total phenolic levels (Fig. S2B).

Generalist Herbivore Performance, Ellagitannin Fractions Diets containing ellagitannin fraction II or ellagitannin fraction IV reduced the performance of the generalist caterpillar compared to control diets (Fig. 2B, Table S4). Control caterpillars had greater mass ($P < 0.001$) and survival ($P < 0.001$, Fig. S1B) than caterpillars fed diet containing mid concentrations of oenothein ellagitannins (5 and 10 mg/g) (Fig. S1B, Table S4). When considering all concentrations at day 5, caterpillars exposed to fraction II (i.e. enriched for oenothein B) had 20% greater mass than caterpillars exposed to fraction IV (i.e. enriched for oenothein A and higher oligomers) ($P = 0.02$; Fig. S2C). At day 10, caterpillars exposed to fraction IV had 40%

greater mass than caterpillars exposed to fraction II, although this effect was only marginally significant ($P = 0.05$). Higher concentrations of both fractions of oenothein ellagitannins led to lower caterpillar mass (Fig. 2B). The effect of ellagitannin fraction and the fraction X concentration interaction were not significant (Table S4). Caterpillars exposed to higher concentrations of either ellagitannin fraction had lower survival ($P = 0.001$, Fig. 3B), with no caterpillars surviving above concentrations of 2.5 mg/g (0.025% ellagitannin fractions). There was no significant effect of ellagitannin fraction or fraction X concentration interaction on the survival of generalist caterpillars (Table S4).

Specialist Herbivore Performance, Total Phenolic Extracts The effects of total phenolics was tested on multiple metrics of performance of the specialist caterpillar *Schinia florida*. Overall, variation in the concentration of total phenolics did not strongly affect caterpillar mass (Fig. 2C). Caterpillar mass did not significantly differ between caterpillars fed control diet versus those fed 5-10 mg/g of phenolics (Table S5). However, survival of caterpillars was 80% on control diets versus 17% on diet containing 5 to 10 mg/g of total phenolics ($P = 0.01$) (Fig. 3C). Phenolic concentration and plant genotype did not affect caterpillar mass or survival when it was treated as a continuous variable, indicating the negative effects of phenolics on the survival of the specialist caterpillar was caused by the clear toxic effects of total phenolic extracts (Table S5). Initial caterpillar mass was a significant predictor for some variables (Table S5).

Specialist Herbivore Performance, Ellagitannin Fractions The specialist *S. florida* was also negatively impacted by the ellagitannin fractions (Fig. 2D, Table S6). Caterpillars feeding on the control diet had greater mass (Table S6) and they experienced higher survival ($P < 0.001$), when compared to caterpillars feeding on diet with 5 to 10 mg/g (i.e. “mid” concentration) of ellagitannin fractions (Fig S1D). The concentration of oenothein ellagitannins had a significant

287 effect on caterpillar mass on day 5, with caterpillars having 45% greater mass in the fraction II
288 treatment when compared to the fraction IV treatment ($P = 0.049$; Fig. 2D). By day 10,
289 caterpillars fed fraction II had only marginally greater mass (24% greater) than those fed fraction
290 IV ($P = 0.07$). There was also a marginally non-significant interaction between fraction and
291 concentration on day 10 ($P = 0.08$), which was caused by proportionally greater caterpillar mass
292 at higher oenothein B concentrations. There were no significant fraction effects on specialist
293 caterpillar mass on day 15, which is likely due to reduced statistical power caused by lower
294 replication; there were concentration effects on caterpillar mass on day 20 ($P = 0.03$, Fig. 2D).
295 Finally, for caterpillar survival there was also a significant interaction between concentration and
296 ellagitannin fraction ($P = 0.04$; Fig. 3D). Specifically, caterpillars experienced greater survival
297 when feeding on diet containing 1 and 5 mg/g of fraction IV compared to caterpillars feeding on
298 fraction II, whereas caterpillars experienced greater survival on the fraction II treatment
299 compared to the fraction IV treatment on day 20 (Fig. 3D). All variables were significantly
300 predicted by initial mass (Table S6).

301 **Discussion**

302 Our results lead to several important conclusions about the role of hydrolysable tannins as
303 defences against insect herbivores, which directly address our research questions. First, we found
304 direct evidence that macrocyclic ellagitannin oligomers have large negative effects on the
305 performance of generalist and specialist insect herbivores (Question 1). Second, differences
306 between fractions rich in smaller or larger oligomers were small and dependent on the insect's
307 life stage (Question 2). Finally, total phenolics had a greater negative impact on the generalist
308 caterpillar, whereas the ellagitannin fractions mostly had a greater negative impact on the

specialist caterpillar (Question 3). Here we discuss these results and compare them to previous work on ellagitannins and herbivore specialization.

Effects of Ellagitannins on Herbivore Performance The evidence for ellagitannins having a direct negative effect on insect herbivore performance is now considerable. It was previously shown that total ellagitannins and the individual ellagitannin compound vescalagin decreased herbivore performance (Barbehenn et al. 2009; Roslin and Salminen 2008). As well, total ellagitannins and the ellagitannin oenothein A have been associated with decreased herbivory using correlative approaches (Agrawal et al. 2012; Anstett et al. 2015; Johnson et al. 2009; McArt et al. 2013). Here we experimentally show that mixtures of oenothein B, oenothein A, and larger oligomers decrease herbivore performance of both one generalist and one specialist herbivorous moth species. The effects of these ellagitannins were large, whereby even small concentrations (0.25-0.5% of tissue dry weight) resulted in large negative and often lethal effects on the generalist and specialist caterpillars (Fig. 2, Fig. 3). Our results provide some of the strongest direct evidence that ellagitannins are an important anti-herbivore defence, and justify their use as a model for understanding the defensive effects of compounds that potentially create oxidative damage, anti-feedant effects or less likely protein precipitation on insects (Appel 1993; Barbehenn et al. 2008; Salminen and Karonen 2011).

Effects of Chemical Defences on Generalist Versus Specialist Herbivores While oenothein A, oenothein B and higher oenothein oligomers are important defences against herbivores, they exist within the context of a wider range of anti-herbivore chemical defences. Although these are potent compounds, it is possible that other compounds, such as polyphenol oxidases, can act in conjunction with ellagitannins to generate higher negative impacts on certain herbivores (Kim et al. 2018). This is true for the generalist *S. exigua*, which showed much lower survival when

feeding on diet with just 1 mg/g of total phenolics (0% survival) compared to an equivalent concentration of the ellagitannin fractions (74% survival: the results from both fractions) (Fig. 3A,B). However, other herbivores may be particularly impacted by just one group of compounds. This was the case for the specialist *S. florida*, which experienced lower survival when feeding on diet with 10 mg/g of the ellagitannin fractions (0% survival: both fractions combined), compared to caterpillars feeding on 10 mg/g total phenolic treatment (33% survival; all genotypes combined) (Fig. 3C, Fig. 3D). However, total phenolics were more harmful at lower concentrations (Fig. 3C; Fig. 3D). Therefore, while the generalist insect may be more impacted by a wider variety of chemical defences, individual compounds may be more effective against the specialist caterpillar *S. florida*, especially if these compounds are at moderate to high concentrations. We interpret these comparisons between *S. exigua* and *S. florida* with an abundance of caution because the methods used for each insect species differed in important ways which may have influenced our results. Moreover, only one generalist and one specialist insect were tested, we caution that it is still unclear if these results are due to differences in diet breadth or differences between the two species unrelated to host specialization.

Limitations There are four limitations to this study which require consideration when interpreting our results. First, *S. florida* were collected from only a single geographic region (Ontario, Canada). *Oenothera biennis* is known to have lower levels of chemical defences in this region, and particularly lower concentrations of oenothein A (Anstett et al. 2015), making it possible that the *S. florida* collected may be less adapted to oenothein A and increased phenolics in general. This issue needs to be further explored by characterizing the genetic diversity of *S. florida* resistance to phenolics and oenothein ellagitannins across multiple regions. Second, *S. florida* were placed into the experiment as second and third instar larvae, rather than as recently

hatched first instar larvae. If it had been possible to use neonate *S. florida* caterpillars, we may have seen higher mortality. We do not think these limitations are a major concern because earlier mortality would likely increase the effects we observed. Third, the oxidative effects of ellagitannins may be increased in the presence of other metabolites and polyphenol oxidases. While this experiment used enriched fractions of ellagitannins placed into diet, previous work performed in vitro still found strong oxidative activity for these purified compound classes (Barbehenn et al. 2006). Additionally, the results of our study agree with previous conclusions from field experiments about the effects of ellagitannins on herbivores, which found oenothein A was associated with decreased insect herbivore damage (Agrawal et al. 2012; Anstett et al. 2015). Finally, the use of an artificial diet formulated for *S. exigua* could have caused nutritional stress for *S. florida* and potentially increased susceptibility to phenolics. This possibility is unlikely to have altered our conclusions because: i) all *S. florida* caterpillars experienced the same base diet; ii) caterpillars were able to complete development on the control diet; and iii) caterpillars showed clear responses to variation in the concentration of ellagitannins. Overall, our results and conclusions are robust to these caveats.

Conclusions Our findings provide clear and compelling support for the defensive function of macrocyclic ellagitannins against generalist and specialist insect herbivores. This study does not find strong evidence that variation in the composition of ellagitannins strongly influences defences against herbivores as previously claimed (Agrawal et al. 2012; Anstett et al. 2015). Future studies should investigate the physiological mechanisms underlying the defensive function of these compounds in insect guts to further characterize the purported effects of oxidative stress, anti-feedancy, and possibly protein precipitation on insect survival and fecundity. Additionally, characterization of the genes involved in the biosynthesis of oenothein

B, oenothein A, and high oligomer ellagitannins would present a major advance in the biochemistry, genetics and evolution of ellagitannin chemistry, and its potential applications.

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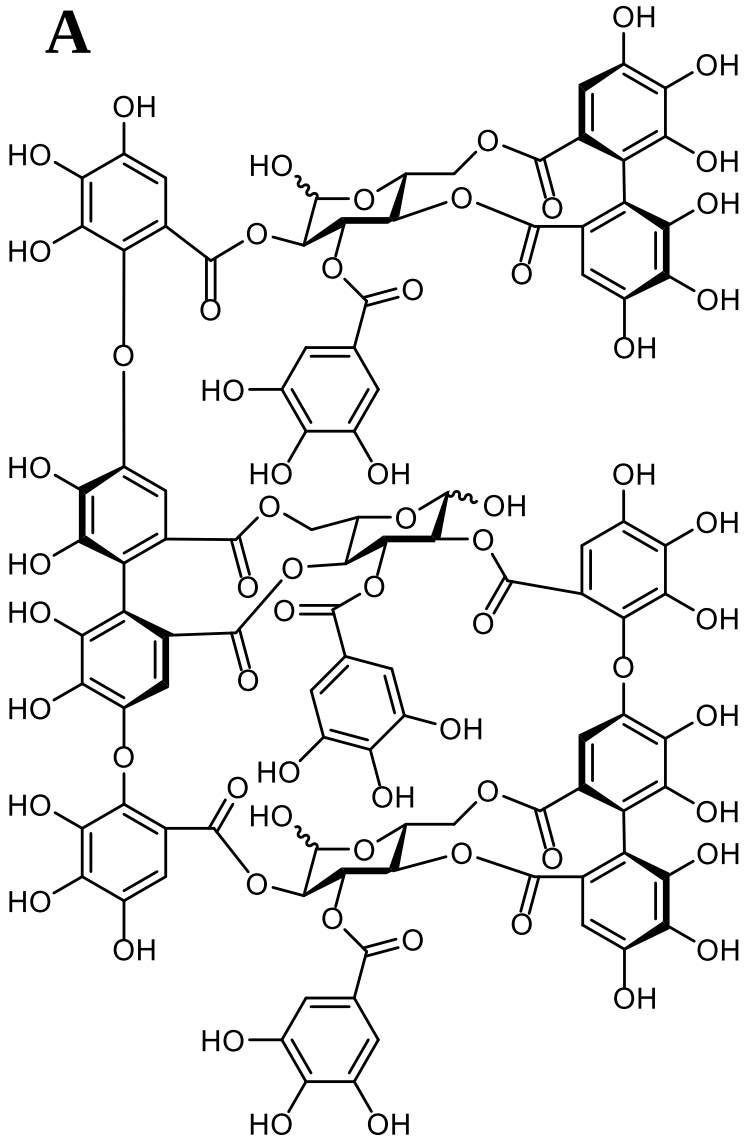
Figure Headings

Fig. 1 Chemical structures of the two dominant ellagitannins present in *Oenothera biennis*. (A) Trimer oenothein A and (B) dimer oenothein B.

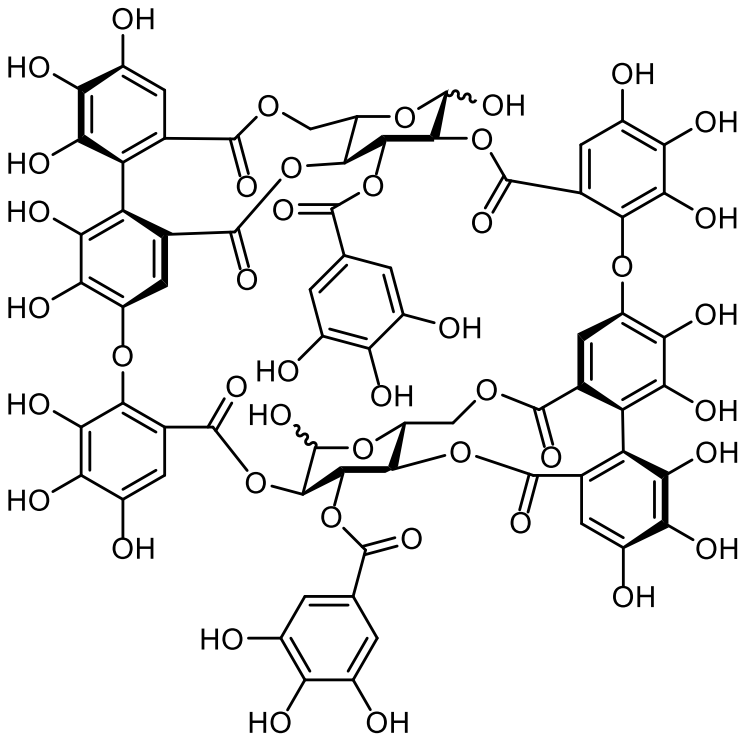
Fig. 2 Time-series of the mass of generalist *Spodoptera exigua* fed diet containing (A) total phenolics or (B) oenothein, and the specialist *Schinia florida* fed diet containing (C) total phenolics or (D) oenothein. Mass is given on a log scale for *S. exigua* and *S. florida* to aid in visualization. Lines represent the best fit line from linear regression. Individual data points have been removed to reduce clutter and make the overall trends clearer. Data are only present up to day 10 for the control, because all caterpillars in this treatment pupated before day 15.

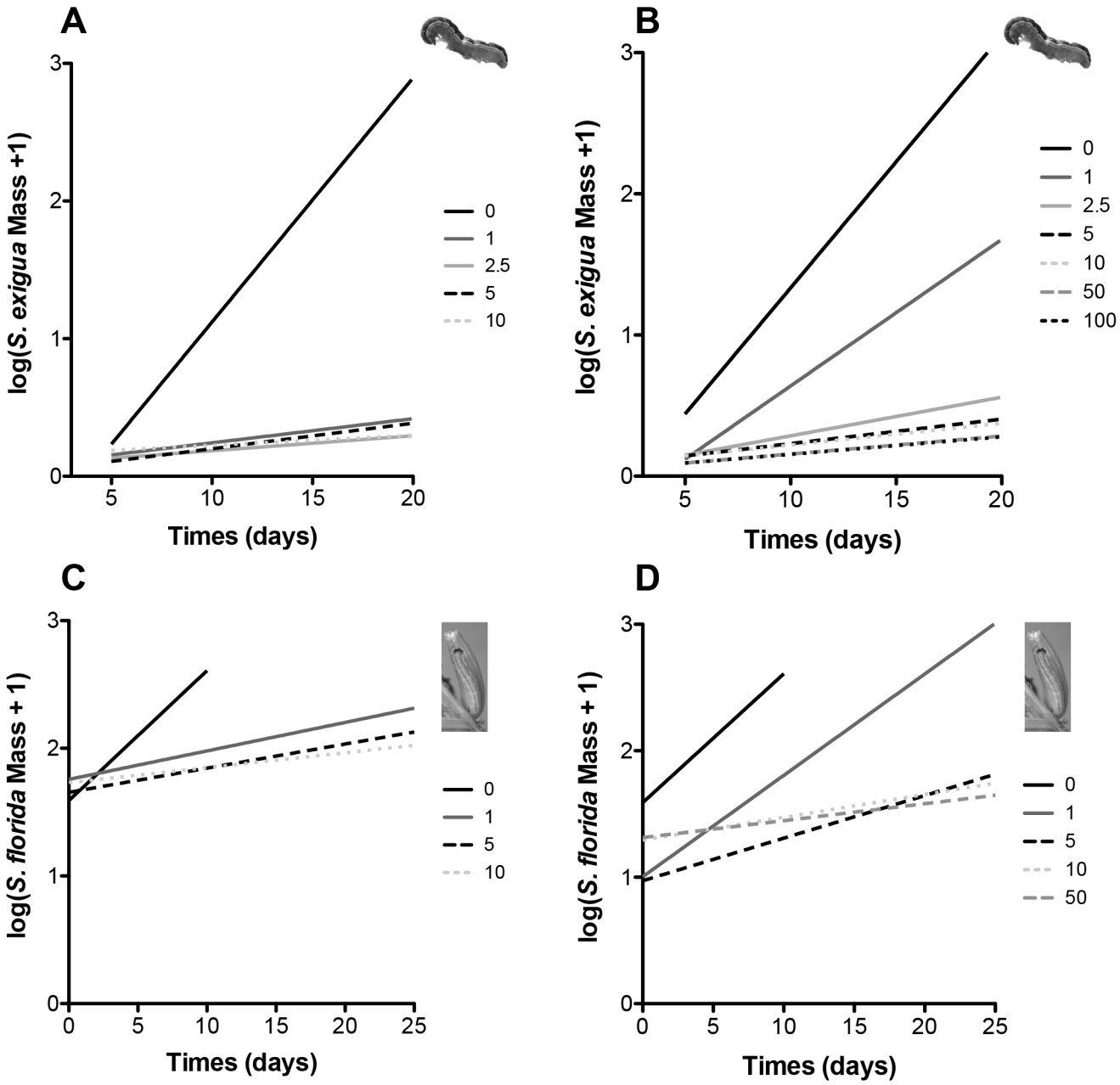
Fig. 3 Survival results for bioassay experiments with generalist *Spodoptera exigua* when fed diet containing (A) total phenolics or (B) oenothein, and the specialist *Schinia florida* fed diet containing (C) total phenolics or (D) oenothein. Each point indicates the mean survival value. Bars indicate standard error. For (D) results are split into two fractions with varying amounts of oenothein A, oenothein B, and higher oligomers (see Table S2). Survival is from the entire experiment is displayed. Total phenolics and ellagitannin fractions are given in dry weight units.

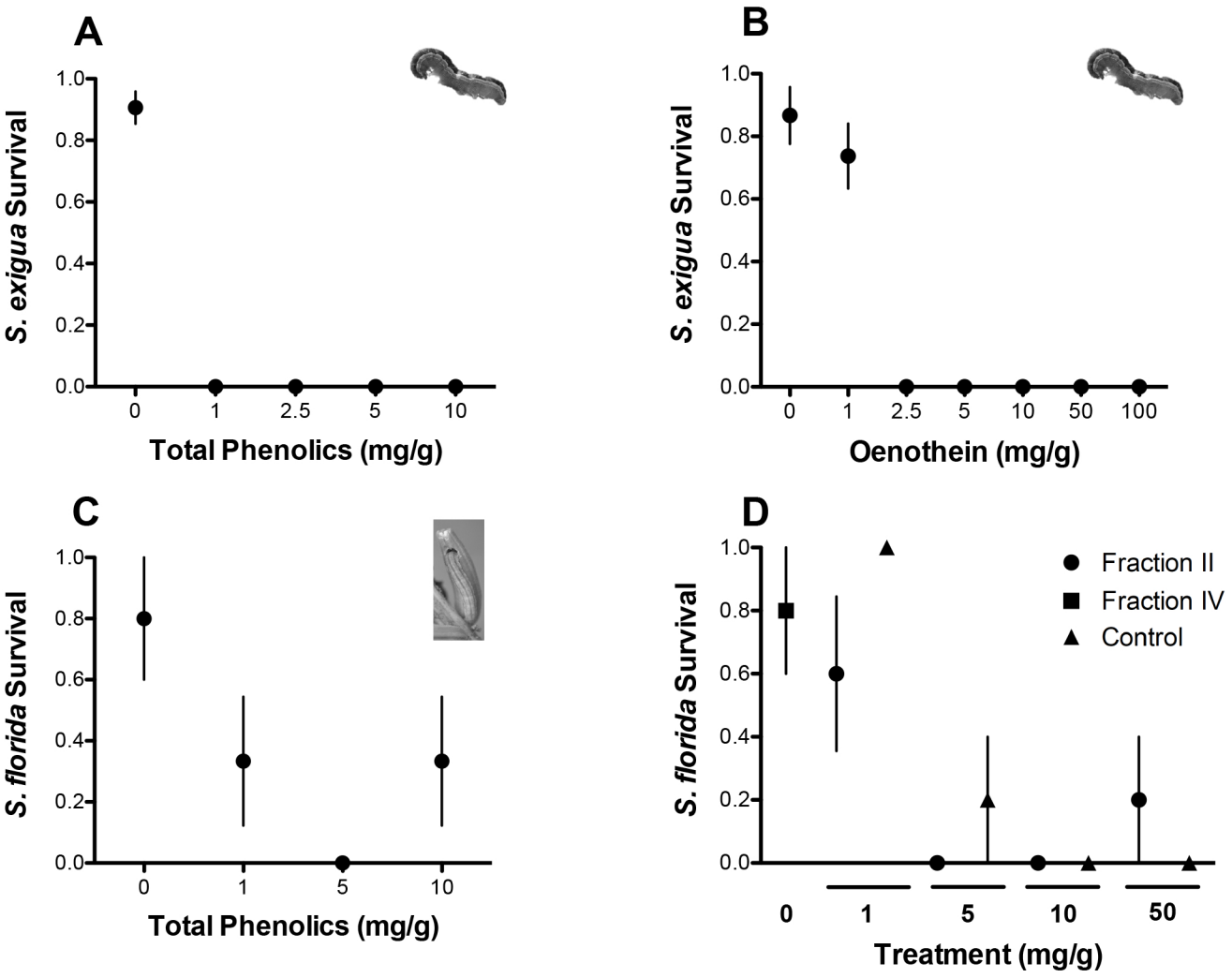
A



B




A
B
C
D
log(S. exigua Mass +1)
log(S. florida Mass + 1)
Times (days)
0
1
2.5
5
10
50
100


A
S. exigua Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2.5
5
10
Total Phenolics (mg/g)
B
S. exigua Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2.5
5
10
50
100
Oenothein (mg/g)
C
S. florida Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
1
5
10
Total Phenolics (mg/g)
D
S. florida Survival
1.0
0.8
0.6
0.4
0.2
0.0
Fraction II
Fraction IV
Control
0
1
5
10
50
Treatment (mg/g)