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Ability of the Oriental Fruit Moth *Grapholita molesta* (Lepidoptera: Tortricidae) to Detoxify Juglone, the Main Secondary Metabolite of the Non-host Plant Walnut

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Abstract Many plant species produce toxic secondary metabolites that limit attacks by herbivorous insects, and may thereby constrain insect expansion to new hosts. Walnut is a host for the codling moth *Cydia pomonella*, which efficiently detoxifies the main walnut defensive compound juglone (5-hydroxy-1,4-naphthoquinone). The oriental fruit moth *Grapholita molesta,* which also belongs to the tribe Grapholitini, does not feed on walnut. We tested the performance of *G. molesta,* a highly invasive species, on artificial diets containing juglone at levels mimicking those found in walnut over the growing season. Juglone-fed *G. molesta* survived relatively well to adulthood, but larval and adult body weights were reduced, and larval developmental time was prolonged in a dose-dependent fashion. Chemical analysis of frass from larvae that had been fed a juglone-containing diet suggests that *G. molesta* reduces juglone to non-toxic 1,4,5-trihydroxynaphthalene in its gut. This unexpected tolerance of *G. molesta* to high levels of juglone may facilitate expansion of the host range beyond the current rosacean fruit trees used by this invasive pest.

Key Words *Grapholita (=Cydia) molesta* · Host range · Invasive species · Juglone · Naphthoquinone · Trihydroxynaphthalene · Toxicity

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Introduction

Host range expansion by herbivorous insects is in many cases limited by plant secondary metabolites. Therefore, adaptation to or tolerance of defensive chemicals by herbivorous species may have important ecological and agricultural consequences (Louda et al., 1997). In Juglandaceae, the toxicity of juglone (5-hydroxy-1,4-naphthoquinone) and other naphthoquinones to numerous lepidopteran species is well known (Yu, 1987; Thiboldeaux et al., 1994; Sun et al., 2007). However, a few insect species, such as the luna moth *Actias luna* (Lepidoptera: Saturniidae) and the codling moth *Cydia pomonella* (Lepidoptera: Tortricidae), include Juglandaceae in their diet. The luna moth and codling moth efficiently metabolize naphthoquinones even at high concentrations (Lindroth, 1989; Piskorski and Dorn, 2011). The luna moth possesses high levels of quinone reductase activity, an enzyme responsible for naphthoquinone detoxification, with highest activity levels measured in midgut microsomes (Yu, 1987; Lindroth, 1989). The codling moth reduces juglone in the gut and excretes non-toxic 1,4,5-trihydroxynaphthalene when it feeds on diets containing juglone (Piskorski and Dorn, 2011).

The oriental fruit moth *Grapholita (=Cydia) molesta* Busck (Lepidoptera: Tortricidae) belongs to the same tribe (Grapholitini) as the codling moth, and both species infest fruit trees of the Rosaceae family that are devoid of naphthoquinones. *Grapholita molesta* uses peach as its primary and apple as a secondary host (Rothschild and Vickers, 1991; Piñero and Dorn, 2009), whereas the codling moth uses apple as its primary host (Phillips and Barnes, 1975). The larvae of both species are internal fruit feeders and are morphologically difficult to distinguish, so that molecular tools may be required to discriminate between

them (Chen and Dorn, 2009). Despite these similarities, infestation by the highly invasive pest *G. molesta* has not been reported on Juglandaceae or other plants that contain naphthoquinones, in contrast to the codling moth, which thrives on walnut (Phillips and Barnes, 1975; Piskorski and Dorn, 2011). Juglone contents in walnut fluctuate over the season (Radix et al., 1998; Colaric et al., 2005; Solar et al., 2006; Stampar et al., 2006), and survival rates of the codling moth were high at the lower juglone level and moderate at the higher level reported for this plant species. Concentrations of juglone that were double the maximum juglone content in walnut were lethal to the codling moth larvae (Piskorski and Dorn, 2011), indicating that detoxification is limited even in a herbivorous species adapted to walnut (Barnes, 1991) and its naphthoquinones. We hypothesized that the oriental fruit moth may be unable to detoxify juglone, thus limiting its potential to use walnut as a host. However, by using performance bioassays we showed that *G. molesta* can survive on a diet containing juglone, and chemical analysis of larval frass suggests the ability of this species to reduce this compound in its digestive tract.

Methods and Materials

Insects Larvae of the oriental fruit moth were collected from a peach orchard in Trentino, Italy, in 2007 and reared in the laboratory under controlled conditions [16 : 8 hL:D cycle at 26/24°C and 60% relative humidity (r.h.)]. Upon emergence, the adults were transferred to a paper cylinder with moist cotton (Hern and Dorn, 1999). Neonate larvae were transferred to Petri dishes and allowed to feed on an artificial diet as specified below. Corrugated cardboard strips were offered for pupation. Unfed first-instars (24± 12 h-old) were used for bioassays.

Artificial Diet The diet offered to larvae was a soya flour-based artificial diet devoid of any fruit material, used in mass rearing of the oriental fruit moth in our laboratory (Torriani et al., 2010). Juglone was added at the end of diet preparation; the diet was homogenized and distributed into plastic Petri dishes of 55-mm diam to yield a 4-mm thick layer (Piskorski and Dorn, 2011). Juglone (purity≥97%; Sigma-Aldrich, Buchs, Switzerland) was added to the diet at concentrations of 5, 25, and 50 mg/g dry weight (d.w.), with the first two concentrations corresponding to the minimum and maximum of the seasonally fluctuating juglone content in walnut husks (Radix et al., 1998; Stampar et al., 2006; Piskorski and Dorn, 2011). The diet was prepared directly prior to conducting the bioassay. Juglone was stable throughout the diet preparation and bioassay (Piskorski and Dorn, 2011).

Effect of Juglone on G. molesta Performance First-instar larvae of *G. molesta* (N=30–34 per juglone concentration) were placed individually with a fine brush into Petri dishes with artificial diet containing juglone or with juglone-free diet (Piskorski and Dorn, 2011). Insects were allowed to develop under controlled conditions with a 16 : 8 hL:D cycle at 26/24°C and 60% r.h. Corrugated cardboard strips were attached to the inner wall of each Petri dish lid after 7 d of feeding to allow for pupation.

Development was monitored daily from day 7 onwards, i.e., before the larvae were expected to terminate feeding prior to pupation. Larval developmental time was recorded as the number of days from introduction of the larva onto the diet until its entry into the cardboard strip for pupation. Pupal developmental time was recorded as the period between larval entry into the cardboard strip and adult emergence, thus including any non-feeding prepupal stage the larva may have undergone (Notter-Hausmann and Dorn, 2010; Piskorski and Dorn, 2011). Larval survival was assessed as the proportion of individuals that entered the cardboard strip in relation to those that had been introduced as first-instar larvae. Pupal survival was assessed as the proportion of emerged adults in relation to the number of individuals that had entered the strip (Notter-Hausmann and Dorn, 2010; Piskorski and Dorn, 2011).

To track sublethal effects, the weight of adults was recorded upon emergence (Velten et al., 2007; Piskorski and Dorn, 2011); within 24 h of emergence the adults were killed by freezing, dried for 24 h in a 60°C oven, and weighed. To determine the larval weight gain, fresh weights of larvae were recorded on the 9th day of feeding (Piskorski and Dorn, 2011), i.e., when all larvae were still feeding and none had yet entered the non-feeding prepupal stage.

Fate of Juglone To follow the fate of juglone in insects feeding on the diet that contained juglone at 25 mg/g d.w. the following samples were analyzed: (1) frass, which was collected only from larvae (N=8), which successfully entered the pupal stage; (2) larval regurgitant (N=12), collected by gently touching the head with a micropipette and collecting the fluid; (3) larval hemolymph (up to 1 µl from each larva; N=12), collected with a micropipette directly from each insect after cutting its cuticle from head to abdomen on the ventral side; (4) fed late-instars, i.e., larvae containing filled guts (N=12); (5) starved late-instars (N=6), which were placed for 17 h in Petri dishes, devoid of any food, on humid filter paper to empty their gut prior to analysis; (6) pupae (5–7 d-old; N=8); (7) newly emerged adults (N=12) (Piskorski and Dorn, 2011). Samples (2)–(5) were collected after a 9-d-feeding period. Samples (1), (2), and (7) (frass, regurgitant, and adults) were taken from insects subjected to the performance trial, whereas samples (3)–(6) (body tissues) were prepared in a separate trial.

Insects were killed by freezing, freeze-dried, and ground before extraction. Samples of each type were pooled. All samples were extracted twice with a $CHCl_3$:MeOH (2:1) mixture (5 ml, or 0.5 ml for regurgitant and hemolymph, 10 min sonication) (Piskorski and Dorn, 2011), the two extracts were combined, and analyzed with gas chromatography–mass spectrometry (GC-MS).

Chemical Analysis GC-MS analysis was performed with a 6890 GC-5973 MS instrument (Hewlett-Packard, Palo Alto, CA, USA) equipped with a 30 m × 0.25 mm i.d., 0.25 μm, DB-5 ms column (J&W Scientific Inc., Folsom, CA, USA). Helium was used as carrier gas at a constant flow (1.0 ml/min); an aliquot of each sample (1 μl) was injected. The oven temperature was kept at 50°C for 2 min, then increased to 320°C at a rate of 10°C/min, and kept at that temperature for an additional 10 min. Electron ionization (70 eV) mass spectra were recorded with ion source temperature of 230°C, and quadrupole temperature of 150°C. Identification of juglone and 1,4,5-trihydroxynaphthalene was based on chromatographic and mass spectrometric characteristics and confirmed with synthetic standards; 1,4,5-trihydroxynaphthalene was synthesized previously in our laboratory (Piskorski and Dorn, 2011). Linear retention indices (RI) were calculated using *n*-alkanes as standards and argon as an unretained compound (Piskorski et al., 2007). If juglone or related compounds were not detected in a given extract, the extract was concentrated and re-analyzed.

Statistical Analysis Developmental times and larval and adult weights were analyzed across treatments using analysis of variance (ANOVA) followed by Tukey's *post hoc* test. The χ^2-test was used to test for differences in the number of surviving individuals between treatments; survival on the control diet served as baseline value. The statistical analyses were performed with the JMP 8.0 software (SAS Institute Inc., Cary, NC, USA).

Results

Effect of Juglone in the Artificial Diet on G. molesta Performance Juglone concentrations reflecting the minimum of the seasonally fluctuating level in walnuts (5 mg/g d.w.) did not have any direct effect on survival rate of *G. molesta,* whereas concentrations reflecting the maximum level (25 mg/g d.w.) significantly affected larval and pupal survival, reducing the number of individuals that reached adulthood by one third compared to the control (Table 1). Amending the diet with a juglone concentration (50 mg/g d.w.) that reflected twice the maximal level reported from the field completely prevented larval development, and all larvae on that diet died within a few days.

A similar effect was observed for larval developmental time (Table 2). Mean developmental time of larvae feeding on diet supplemented with juglone at a concentration representing the lowest natural levels was not affected. Significantly prolonged development time of larvae fed on diets containing the highest juglone level reported from the field was observed. It lasted nearly twice as long as in the control. Pupal developmental time

Table 1 Percent survival of *Grapholita molesta* reared on the artificial diet containing juglone

Diet	N[a]	Developmental stage	Actual survival rate to the stage[b]	Apparent survival rate during the stage[c]	P[d]
Juglone 5 mg/g	34	Larva	100	94	ns
	32	Pupa	94	97	ns
	31	Adult	91	–	–
Juglone 25 mg/g	32	Larva	100	66	***
	21	Pupa	66	86	*
	18	Adult	56	–	–
Juglone 50 mg/g	32	Larva	100	0	***
	0	Pupa	0	0	–
	0	Adult	0	–	–
Control (= basis for comparison)	30	Larva	100	90	–
	27	Pupa	90	96	–
	26	Adult	87	–	–

[a] The number of individuals entering a given developmental stage

[b] The percentage of individuals that successfully entered the stage in relation to those that had been introduced as first-instar larvae

[c] The percentage of individuals that successfully entered the next developmental stage in relation to those in the respective stage

[d] ns $P \geq 0.05$; * $P < 0.05$; *** $P < 0.001$ (comparison of apparent survival rate with χ^2-test; survival on the control diet served as baseline value)

Table 2 Developmental time of immature stages of *Grapholita molesta* reared on artificial diets containing juglone

	Larva (d)[a]	Pupa (d)[a]
Juglone 5 mg/g	11.7±0.3 (32) a	10.4±0.1 (31) a
Juglone 25 mg/g	19.9±0.9 (21) b	11.1±0.2 (18) a
Juglone 50 mg/g[b]	–	–
Control	10.6±0.2 (27) a	10.1±0.1 (26) a

[a] Mean values±SEM of the number of individuals given in brackets. Different letters after the mean indicate significant differences between treatments in a column at $P<0.05$ (comparison between treatments with ANOVA and Tukey's *post hoc* test)

[b] No development at the larval stage

remained unaffected at both of these juglone concentrations (Table 2).

Sublethal effects of juglone on insect weight were noted. After 9 days, larvae feeding on diets containing juglone had significantly lower weights compared to larvae feeding on the control diet (Table 3). Larval weight was reduced only about 10% by the low juglone diet, but was reduced 95% by levels of juglone similar to the highest levels reported in walnut. Adult weights were reduced approximately 25% for larvae fed diets containing the high level of juglone reported from walnut husks (Table 3).

Fate of Juglone To understand the successful development of *G. molesta* feeding on diet containing juglone, chemical analysis of larval exudates, hemolymph, and insect bodies was performed in search of juglone or its likely metabolites. In larval frass, the reduced form of juglone, 1,4,5-trihydroxynaphthalene, was detected in relatively high concentrations compared to juglone, with a ratio of 1.22 : 1.00 (1,4,5-trihydroxynaphthalene : juglone). In fed larvae, the guts contain food and digesta, and traces of both juglone and its reduced form were detected. In starved larvae, the gut is empty and neither juglone nor its reduced form were present. Furthermore, neither these nor related phenolic or quinone derivatives were detected in larval regurgitant, in larval hemolymph, in pupae, or in adults. Other likely derivatives of juglone were not detected in any of the samples.

Discussion

The oriental fruit moth *G. molesta*, which has not been reported to use plants containing naphthoquinones as hosts, successfully achieved adulthood on diets supplemented with juglone at levels reflecting those found in walnut husks. At juglone concentrations reflecting the minimum and maximum of the seasonally fluctuating levels (Radix et al., 1998; Stampar et al., 2006), survival rates to adulthood were 90% and more than 50%, respectively, of the control survival rate. Survival rates observed in an analogous experiment with the codling moth *C. pomonella*, which readily accepts walnut as a host, were similar at the low juglone concentration, and only approximately 20% higher at the high juglone concentration (Piskorski and Dorn, 2011).

Sublethal effects observed in juglone-fed *G. molesta* consisted of slightly reduced larval weight gain at the low juglone concentration, and prolonged developmental time as well as reduced larval and adult weight at the high juglone concentration as compared to the control. These effects qualitatively resemble those found recently for *C. pomonella* (Piskorski and Dorn, 2011), but they are quantitatively more pronounced in *G molesta*. Development was completely prevented in *G. molesta* at the juglone level reflecting twice the maximum reported from the field, suggesting that the adverse effects were dose dependent. Similarly, none of the *C. pomonella* larvae exposed to this excessively high level of juglone survived (Piskorski and Dorn, 2011), indicating that even in a herbivorous species that includes naphthoquinone-containing plant material in its diet, the capability to cope with these defensive metabolites is limited.

It is surprising that an herbivorous species can tolerate a defensive compound that is not a constituent of its reported hosts. In fact, physiological constraints of plant-feeding insects are thought to be particularly serious limitations to host plant expansion (Praz et al., 2008; Sedivy et al., 2011). Similar to the oriental fruit moth, the fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae) and the gypsy moth *Lymantria dispar* (Lepidoptera: Lymantriidae) have not been observed to feed on plants that contain naphthoquinones. Juglone is lethal for these two lepidopteran larvae at doses as low as 2.0 mg/g (Yu, 1987; Lindroth et al., 1990), which is lower than the lower limit reported for walnut (Radix et al., 1998; Stampar et al., 2006;

Table 3 Larval fresh weight after 9 days of feeding and adult dry weight of *Grapholita molesta* reared on artificial diet containing juglone

	Larva (mg)[a]	Adult (mg)[a]
Juglone 5 mg/g	14.6±2.1 (25) b	3.9±0.2 (31) a
Juglone 25 mg/g	0.9±0.3 (17) c	3.1±0.3 (18) b
Juglone 50 mg/g[b]	–	–
Control	16.1±1.3 (20) a	4.1±0.2 (26) a

[a] Mean values±SEM of the number of individuals given in brackets. Different letters after the mean indicate significant differences between treatments in a column at $P<0.05$ (comparison between treatments with ANOVA and Tukey's *post hoc* test)

[b] No development at the larval stage

Piskorski and Dorn, 2011), and lower than the concentrations tolerated by the oriental fruit moth.

The mechanism of tolerance to juglone by the oriental fruit moth needs further investigation. In other lepidopteran species, redox cycling (Thiboldeaux et al., 1994) or conjugation with glutathione (Thiboldeaux et al., 1998) could not satisfactorily explain the observed effects of 1,4-naphthoquinones. However, enzymatic reduction of the conjugated cyclic 1,4-dione moiety to the corresponding diol offered a good explanation for different susceptibilities of various lepidopteran species. Quinone reductase, which catalyses reduction of juglone, is active in the intestinal system of some lepidopterans (Yu, 1987; Lindroth, 1989). Low levels of quinone reductase activity (maximum 50 nmol/min/mg protein) were found in the fall armyworm and the gypsy moth, which perform poorly on juglone-containing diet (Yu, 1987; Lindroth et al., 1990). In contrast, a high level of quinone reductase activity (174 to 288 nmol/min/mg protein) was measured in the luna moth, which performs well on juglone-containing diets (Lindroth, 1989).

We suggest that juglone is reduced in the intestinal system of *G. molesta*. Larval frass of *G. molesta* contained relatively high amounts of the trihydroxynaphthalene, but also some juglone, that may represent the compound derived directly from the diet. However, it is more likely that juglone is the product of a spontaneous reoxidation of 1,4,5-trihydroxynaphthalene in air (Hedin et al., 1980; Piskorski and Dorn, 2011). Various plant phenolic compounds are oxidized in the insect's gut (Summers and Felton, 1994; Barbehenn et al., 2003), but such reoxidation of trihydroxynaphthalene seems to be negligible, as indicated by the current work and two previous studies on juglone-tolerant lepidopterans (Lindroth, 1989; Piskorski and Dorn, 2011). Moreover, the absence of juglone in hemolymph and in larvae with emptied guts indicates that this compound is not sequestered by the insect. Hence, the mechanism of overcoming naphthoquinone-based plant defense in *G. molesta* may resemble that recently described for *C. pomonella* (Piskorski and Dorn, 2011).

Lack of infestation of Juglandaceae by the invasive *G. molesta* may have various reasons. First, the volatile profile of walnut (Bezemer and Mills, 2001; Witzgall et al., 2005; Casado et al., 2008) differs from that of peach, this herbivore's primary host (Natale et al., 2003) and from that of apple, its secondary host (Vallat and Dorn, 2005). Hence, the volatile blend emitted from walnut may fail to attract oriental fruit moth females during flight. Second, deterrent properties of naphthoquinones may prevent host acceptance, as shown for other insect species (Ferkovich and Norris, 1971; Faccoli et al., 2005). Third, the skin of walnut husks may pose a harder physical barrier than that of peach and other rosacean fruits, thus preventing penetration by neonate larvae. Finally, we cannot exclude that some infestations may have gone unnoticed, as has similarly been discussed for related internal fruit feeders (Chen and Dorn, 2009).

We do not know whether juglone influences other life-history traits in this herbivore, such as fertility, mating success, longevity, or flight capacity (Cisneros and Barnes, 1974; Keil et al., 2001; Moreau et al., 2006; Torriani et al., 2010). Lifetime fecundity may suffer in cases with reduced adult weight (Bezemer and Mills, 2001) as noted for the high, but not for the low juglone level reported from walnut husks. However, naphthoquinones can be antimicrobial (Clark et al., 1990; Inbaraj and Chignell, 2004) and can act as dietary antioxidants (Johnson and Felton, 2001; Rodríguez et al., 2007), so that by feeding on nutritional sources containing naphthoquinones, insect herbivores may compensate for some fitness losses, or even gain fitness benefits by being better protected against infections and strong oxidants.

It is commonly accepted that many herbivores can widen not only their geographic but also their host range (Dambroski et al., 2005; Myers et al., 2006; Erbout et al., 2009; Chen and Dorn, 2010; Piskorski et al., 2010). The European corn borer *Ostrinia nubilalis* (Lepidoptera: Pyralidae) successfully added maize to its original host range in spite of the toxic properties of hydroxamic acids from this plant, in particular 2,4-dihydroxy-7-methoxy-l,4-benzoxazin-3-one (DIMBOA) (Campos et al., 1988; Martel et al., 2003). Likewise, apple was initially reported only as a secondary, relatively unimportant host plant of the oriental fruit moth (Rothschild and Vickers, 1991), but its significance as a host is continuously increasing (Natale et al., 2004; Myers et al., 2006), indicating the potential of this insect to rapidly adapt to new hosts. Our results suggest that in future, *G. molesta* could include juglandacean plants into its host range, provided other factors are favorable.

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