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Survival physiology and sex ratio of the Chinese white pine beetle *Dendroctonus armandi* (Coleoptera: Scolytinae) during host colonization and overwintering

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Abstract

The Chinese white pine beetle *Dendroctonus armandi* (Coleoptera: Scolytinae) typically displays bivoltinism at altitudes below 1700 m in the Qinling Mountains, China. The periods of host colonization and larval overwintering are two important phases in the life cycle of bark beetles, as it is during these periods that they have to contend with host plant defences and periods of intense cold, respectively. Although during different seasons, the females and males of Chinese white pine beetles show varying tolerances to host plant terpenoids, the sex ratio and survival physiology condition of the two beetle generations are unknown. We investigated the sex ratio of individuals, and also examined the body mass, energy stores, and detoxication enzymes of males and females in each of the two generations in order to determine the overall population stability of each generation. We identified a female-biased sex ratio among adults in both generations. Furthermore, patterns of body mass, energy stores, and detoxication enzymes were found to differ between the two sexes and two seasons. Compared with the males, the females have a larger body mass and higher amounts of stored lipids, which are assumed to be adaptations designed to overcome host resistance and facilitate subsequent oviposition.

Keywords: *Dendroctonus armandi*, sex ratio, energy stores, detoxication enzymes, host colonization

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Introduction

Beetles of the genus *Dendroctonus* have an ancient association with conifers (Sequeira *et al*., 2000), and apart from a brief dispersal period during which the adults locate new host trees, they complete the majority of their life cycle under the bark of conifer trees (Dai *et al*., 2015). The Chinese white pine beetle *Dendroctonus armandi* (Coleoptera: Scolytinae) is arguably the most destructive forest insect in the Qinling Mountains, Shaanxi, China (Yin *et al*., 1984; Chen & Yuan, 2000, 2002). This native pest often reaches epidemic proportions and causes widespread mortality of trees in both natural and managed forest ecosystems. *Dendroctonus armandi* primarily attacks healthy Chinese white pine (*Pinus armandi*) trees aged 30 years or more (Chen & Tang, 2007; Chen *et al*., 2010).

The periods of host colonization and larval overwintering are two important phases in the life cycle of bark beetles, as it is during these periods that they have to contend with host plant defenses and periods of intense cold, respectively (Huber &

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Robert, 2016). At sites under 1700 m in altitude in the Qinling Mountains, *D. armandi* typically displays bivoltinism (Chen & Tang, 2007). Females are earlier to emerge than males and proceed to bore through the bark of the host tree. The later emerging males are dependent on female aggregation pheromone attractants for colonization and reproduction (Li & Zhou, 1992). With regards to colonization, it has previously been found that both the females and males of *D. armandi* show varying tolerances to host terpenoids during different seasons (Dai *et al*., 2015).

Scolytine beetles, such as the mountain pine beetle (*Dendroctonus ponderosae*), generally exhibit female-biased sex ratios (Reid, 1958; Cole *et al*., 1976; Amman & Cole, 1983; Lachowsky & Reid, 2014), which has important consequences with regards to its influence on effective population sizes and population growth rates (James *et al*., 2016). However, skewed sex ratios may arise if there is sex-biased mortality during development (Lachowsky & Reid, 2014). Such sex-biased developmental mortality could be attributable to the body size of sexual size dimorphic beetles, as body size can affect mortality particularly in response to stressors such as cold temperature in winter (Lachowsky & Reid, 2014) and host chemical defenses (Reid & Purcell, 2011; Dai *et al*., 2015). Moreover, if accounting for body weight, sex did not have a significant effect on the survival with most of the monoterpenes for *D. ponderosae* (Chiu *et al*., 2017).

Moreover, sexual differences in physiological characteristics such as energy stores and detoxication enzymes are factors that can have a significant influence on beetle reproduction and population stabilization. Insects store energy reserves in the form of glycogen and triglycerides in adipocytes, the main fat body cells (Arrese & Soulages, 2010). Glycogen is a polymeric form of glucose that can be readily degraded on demand to be used as a glycolytic fuel (Steele, 1982), and in insects it is mobilized in the form of trehalose for overcoming cold temperatures (Storey, 1997; Thompson, 2003). Triglycerides are the stored form of fatty acids, which can be used for energy production through β-oxidation (Athenstaedt & Daum, 2006). Fatty acids serve as precursors in the synthesis of eicosanoids and pheromones (Lockey, 1988; Stanley, 2006). Furthermore, insects can also mobilize and/or utilize fatty acids stored in the lipid droplets of fat bodies for physiological processes such as flight, synthesis of trehalose and proline, and enduring starvation (Arrese & Soulages, 2010).

The resistance of insects to xenobiotics through the activity of three major classes of detoxifying enzymes [cytochromes P450 (CYP), glutathione-*S*-transferases (GSTs) and carboxylesterases], is an ideal system for studying the processes of microevolution and environmental adaptation (Li *et al*., 2007). GSTs are also multifunctional enzymes that conjugate xenobiotic compounds with a glutathione moiety (GSH) and often work in tandem with cytochromes P450 or other enzymes that aid in detoxification, sequestration, or excretion of toxic compounds (Jakoby & Ziegler, 1990; Sheehan *et al*., 2001; Paumi *et al*., 2004; Gunasekaran *et al*., 2011). Furthermore, in insects within the orders Hymenoptera, Lepidoptera, and Diptera, esterases are associated with resistance to pesticides (Li *et al*., 2007).

Previous research on the Chinese white pine beetle has shown that adult females and males from different seasons have different tolerances to host plant terpenoids (Dai *et al*., 2015), and that overwintering larval survival is influenced by a number of physiological factors, including the size of energy store (Wang *et al*., 2017). In *D. armandi*, several genes of the three major classes of detoxifying enzymes play important roles in detoxification related to their specific behavior and development (Dai *et al*., 2015, 2016). In this study, we investigated the sex ratio of the two seasons of *D. armandi*, and also examined the body mass, energy stores, and detoxication enzymes of adult males and females from the two generations, in order to determine if they contribute to population stabilization of each season's adults.

Materials and methods

Insect collection

The early season adults, whom develop from overwintering larvae, appear in May, and following colonization and mating, produce the late season (summer) adults which emerge before September.

Dendroctonus armandi individuals from the two seasons were collected from infested *P. armandi* growing on the southern slopes of the middle Qinling Mountains, Shaanxi, China (33°18′N, 108°21′E). As a source of beetles for study, we selected two infested *P. armandi* trees (checking the emergence hole to make sure the tree was infested with only one season beetles) of similar age (over 30 years and diameter ∼20 cm) during each season in 2016. The *P. armandi* that was first infested by beetles at the autumn of the previous year was selected at early May to collect the early season adults. And the late season adult beetles were collected from the *P. armandi* that was newly infested in the summer. Prior to beetle eclosion, we felled down the trees, which were subsequently sawn into 1.3-m-long logs. All logs were transferred to the laboratory and maintained under nylon nets for beetle collection. In total, we collected approximately 1000 emerged adults (both females and males) in each generation.

Body mass and sex ratio

Upon collection, the body mass of each adult was immediately measured using an electronic balance ($d = 0.0001$ g, Tianjin, AL204; Mettler-Toledo Ltd., China). Thereafter, the individuals were sexed according to the shape of the seventh abdominal tergite (Lyon, 1958) and then stored at −20 or −80°C for subsequent physiological and molecular biological experiments, respectively.

Energy stores

For both sexes in each generation, we measured three physiological indices, namely the content of glycogen, triglycerides, and free fatty acids, using appropriate biochemical methods. Measurements for each index were obtained from five biological replicates (five beetles for one replicate).

Glycogen content was measured from males and females according to Van Handel & Day (1988), using the hot anthrone method (Van Handel, 1985; Chen *et al*., 2010). Triglycerides were extracted from males and females in chloroform/methanol using the method described by Folch *et al*. (1957) and quantified as described by Patel *et al*. (2011). Free fatty acids were quantified using a kit following the manufacturer's recommendations (MAK044; Sigma-Aldrich, Inc., USA). Absorbances were determined using a UV-1800PC spectrophotometer (Shanghai Mapada Instrument Co., Ltd. China), and the photometric readings were converted into milligrams or nanomoles per fresh weight (g).

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Table 1. Two-way analysis of variance (ANOVA) results of energy store and detoxication enzymes in two generation adults of *D. armandi.*

	df		Glycogen	Triglyceride	Free fatty acid	Reduced glutathione	Carboxylesterase	Cytochrome b5
Sex	1	*F*	0.281	0.744	26.911	2.174	58.747	6.993
		P-value	0.603	0.401	**<0.001**	0.160	**<0.001**	**0.018**
Season	1	*F*	100.043	1.959	3.895	339.766	63.531	0.683
		P-value	**<0.001**	0.181	0.066	**<0.001**	**<0.001**	0.421
S*S	1	*F*	0.083	40.556	0.401	1.108	137.549	0.031
		P-value	0.777	**<0.001**	0.536	0.308	**<0.001**	0.862

Bold fonts indicates significant difference between sexes, seasons and S*S interaction with Two-way ANOVA ($\alpha = 0.05$).

Detoxication enzymes

We also measured cytochrome b5 content, reduced glutathione content, and carboxylesterase activity in both males and females of each generation to analyze difference in the detoxication enzymes between sexes and generations. For each determination, measurements were obtained from five biological replicates, each of which comprised five beetles of pre-determined body mass.

Cytochrome b5 content was determined using the method of Takeshita *et al*. (1980). The standard assay for GSH content was carried out under conditions similar to those described by Smith & Anderson (1992). Carboxylesterase activity was measured using α-Naphthol as a standard according to the method described by Van Asperen (1962). Absorbance were determined using a UV-1800PC spectrophotometer (Shanghai Mapada Instrument Co., Ltd. China), and the photometric readings were converted to nanomoles or activity unit per fresh weight (*g*). All chemicals used in this study were chemically pure and purchased from Tianjin Kemiou Chemical Reagent Co., Ltd., China.

In addition, we also examined the transcription levels of 19 P450 genes and *DarmCyt-b5* in these two season adults. The experimental procedure was performed as previously described (Dai *et al*., 2015). For each sex and season, the total RNA used for real-time qPCR was obtained from three biological replicates, and each biological replicate contained three beetles.

Statistical analysis

A binomial distribution test was used to assess the sex ratio of adults in each generation. And the significant difference of sex ratio between the two generations was performed with Pearson's χ^2 test. Whereas Mann–Whitney tests were used to analyze the differences in body mass between males and females from the two generations (Dinneen & Blakesley, 1973), since the body mass of each sex and season (except males of early season) were not in accordance with normal distribution. Two-way ANOVA with sex and season as fixed factors was used to analyze the differences in energy store and detoxication enzymes in the Chinese white pine beetles.

Relative expression of Chinese white pine beetles' 19 CYPs and *DarmCyt-b5* was determined using the Ct (ΔΔCt) method (Livak & Schmittgen, 2008). The fold changes in expression between two sexes (males' relative expression to females) of each season were evaluated using the $2^{-\Delta\Delta Ct}$ values. And the fold change values were $\log_2$ transformed for statistical analyses and plotting.

In all cases, significance was indicated at the 5% level. All statistical analyses were performed using SPSS 18.0 (IBM SPSS Statistics, Chicago, IL, USA) and plotted using SIGMAPLOT 12.0 software (Systat Software Inc., San Jose, CA, USA).

Results

Body mass and sex ratio

For both generations, we found a greater number of females than male beetles, with a male percentage of 45.8% (n = ♀522 + ♂441) for the early season and 39.7% (n = ♀600 + ♂395) for the late season. The binomial distribution test indicated a skewed sex ratio in both generations (early season, $P = 0.010$; late season, $P < 0.001$). Furthermore, compared with early season adults, a greater proportion of late season adults were female (Pearson's chi-squared test: $\chi^2 = 7.433$, $df = 1$, $P = 0.006$).

The median body mass of early season females and males was 0.0088 ± 0.0016 and 0.0084 ± 0.0016 g, respectively, whereas that for late season females and males was 0.0090 ± 0.0019 and 0.0086 ± 0.0018 g, respectively. There were significant differences in the body mass of adult males and females in each season (Mann-Whitney *U* test: early season, Mann-Whitney $U = 101{,}980.50$, $Z = -3.052$, $P = 0.002$; late season, Mann-Whitney $U = 105{,}230.00$, $Z = -2.992$, $P = 0.003$), and we also detected a significant difference in body mass between early and late season adult females (Mann-Whitney $U = 141{,}293.50$, $Z = -2.828$, $P = 0.005$).

Energy stores

Although, we identified no significant difference in the glycogen content between sexes ($df = 1$, $F = 0.281$, $P = 0.603$), we detected significant between-season differences ($df = 1$, $F = 100.043$, $P < 0.001$) (table 1). For both males and females, early season beetles had higher glycogen content than late season individuals (fig. 1a). In contrast, although triglyceride content was higher in early season females, it was higher in late season males (fig. 1b). The significant difference was only found in the interaction of seasons and sexes ($df = 1$, $F = 40.556$, $P < 0.001$) (table 1). In both seasons, females were found to have a significantly higher free fatty acid content than males ($df = 1$, $F = 26.911$, $P < 0.001$) (table 1 and fig. 1c). Significant differences in free fatty acids was no detected between season and the interaction of seasons and sexes (Table 1).

Detoxication enzymes

Females had significantly higher ($df = 1$, $F = 6.993$, $P = 0.018$) cytochrome b5 content than males (fig. 1d). There was no significant difference between seasons and S*S interaction for cytochrome b5 content (table 1). Late season adults did, however, have significantly higher levels of reduced glutathione compared to early season adults ($df = 1$, $F = 339.766$, $P < 0.001$) (fig. 1e). No significant differences

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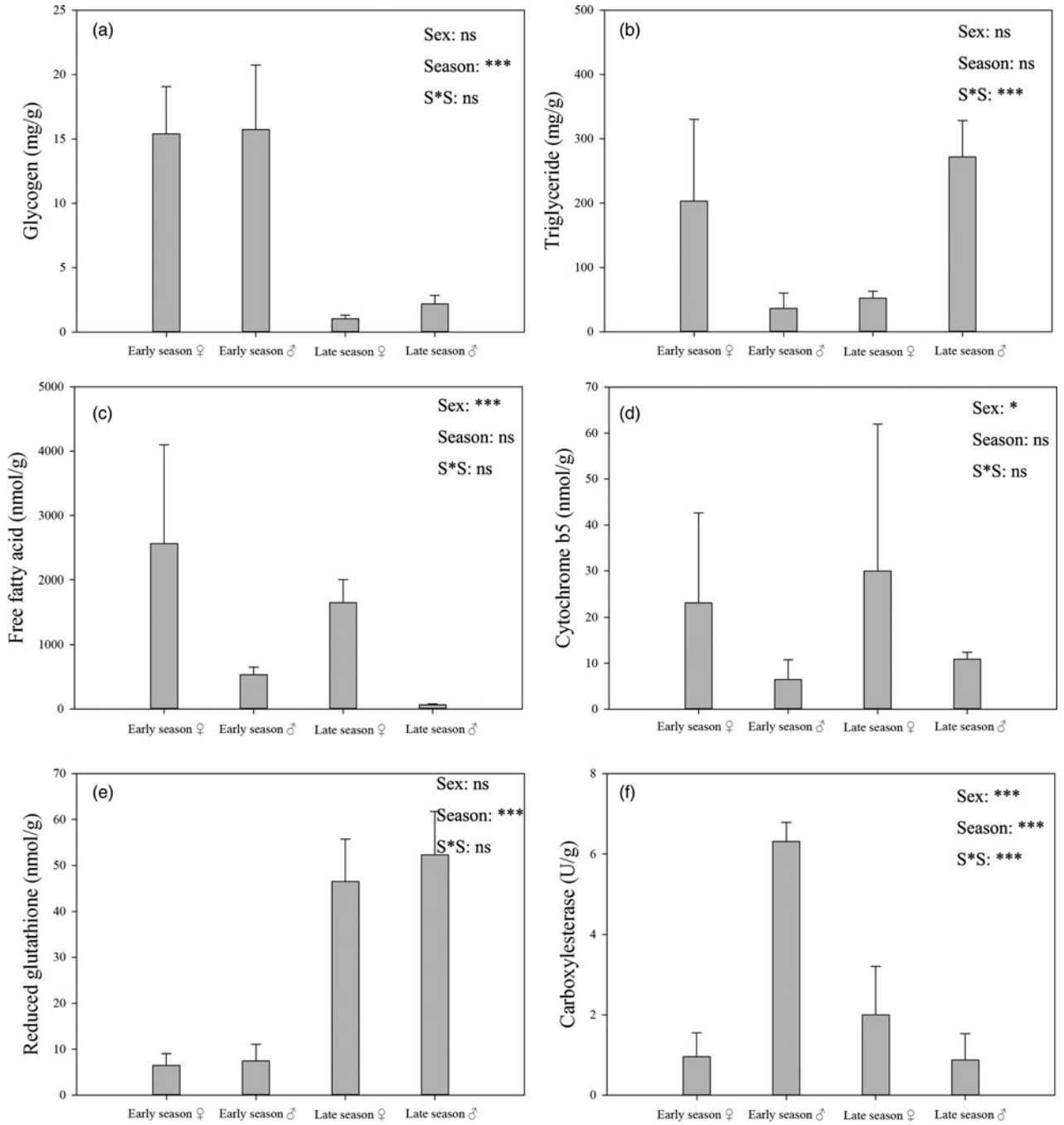

Fig. 1. Content of glycogen (a), triglyceride (b), free fatty acid (c), cytochrome b5 (d), reduced glutathione (e) and carboxylesterase (f) activity in two different generation adults of *D. armandi*. Values are presented as the mean ± SE. Measurements for each index were obtained from five biological replicates (five beetles for one replicate) ($N = 5 \times 5$). The significant difference between sexes, seasons and S*S interaction with Two-way ANOVA (ns, no significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

between sex and S*S interaction were found for reduced glutathione (table 1). The significant differences were found between sexes, seasons and their interaction for carboxylesterase activity (table 1). Carboxylesterase activity was significantly higher in early season males than females, whereas lower in late season males than females (fig. 1f).

We used specific primers of 19 P450 genes and *DarmCyt-b5* in order to determine their transcript level abundance between sexes in early and late season *D. armandi* adults. Significant differences were found from 9 P450 genes' transcript levels in the early season adults, but 14 P450 genes in late season adults (table 2). We identified variations in P450 gene transcript

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Table 2. One-way analysis of variance (ANOVA) results of cytochrome P450 (CYP) genes and *Darmcyt-b5* transcript levels between sexes in two season adults of *D. armandi*.

		Early season		Late season	
Genes	*df*	*F*	*P*-value	*F*	*P*-value
CYP302A1	1	0.007	0.937	9.117	**0.039**
CYP305F1	1	6.114	0.069	0.525	0.509
CYP307A2	1	110.061	**<0.001**	96.439	**0.001**
CYP334E1	1	221.261	**<0.001**	355.932	**<0.001**
CYP345E4	1	84.195	**0.001**	50.356	**0.002**
CYP345F1	1	65.402	**0.001**	25.315	**0.007**
CYP347E1	1	3.855	0.121	2.394	0.197
CYP349B2	1	11.894	**0.026**	64.422	**0.001**
CYP393A1	1	7.822	**0.049**	249.444	**<0.001**
CYP4BD7	1	6.03	0.070	43.699	**0.003**
CYP4EX1	1	13.054	**0.022**	194.076	**<0.001**
CYP6BX1	1	0.06	0.819	11.19	**0.029**
CYP6DF1	1	3.867	0.121	4.069	0.114
CYP6DG1	1	1.182	0.338	131.868	**<0.001**
CYP6DH5	1	1.675	0.265	120.827	**<0.001**
CYP6DJ2	1	106.68	**<0.001**	283.549	**<0.001**
CYP9AN1	1	7.961	**0.048**	1.409	0.301
CYP9Z18	1	6.916	0.058	2.788	0.170
CYP9Z20	1	1.174	0.339	29.397	**0.006**
Darmcyt-b5	1	0.295	0.616	1.522	0.285

Bold fonts indicates significant difference between sexes of the same season with One-way ANOVA ($\alpha = 0.05$).

levels in the males and females (fig. 2). Compared with the females, 6 P450 genes were found to be less abundant in males in early season, and 12 in late season (fig. 2). There were two special P450 genes: CYP334E1 and CYP4EX1, which were more abundant in males compared with females in both seasons (fig. 2). Whereas for both seasons, no significant difference in the transcription levels of *DarmCyt-b*5 was detected between males and females (Table 2).

Discussion

For both the generation adults of Chinese white pine beetles, we detected a clear skew in the sex ratio, although this was more pronounced in the late season adults. We assume that the female-biased sex ratio in *D. armandi* is a consequence of interactions between male beetles and their environment and, as is also the case from mountain pine beetle, not due to an adverse response of adult females during oviposition (Lachowsky & Reid, 2014; James *et al.*, 2016).The higher rate of male mortality relative to females was not only due to overwintering temperature stress (Lachowsky & Reid, 2014), as the skew in sex ratio was more pronounced in the late season adults that develop from non-overwintering larvae. It is possible that male larvae might suffer higher host defense-related mortality in summer compared with overwintering losses. Moreover, there is no evidence that shows an unequal sex ratio at oviposition in bark beetles.

To ensure survival, both season females and males must contend with different environmental pressures; for example, different host conditions, rapid changes in temperature, and unsettled weather conditions. The body mass, energy stores, and detoxication enzyme levels of adult beetles from different seasons are assumed to determine their success in host colonization and reproduction.

The higher fatty acid and triglyceride content in females than in males might be related to oviposition, as the vast majority of lipid accumulates in the oocytes and is transported to the ovaries by lipophorin (Ziegler & Ibrahim, 2001; Ziegler & Van Antwerpen, 2006). Triglycerides are a stored form of fatty acids that can be used for energy production via β-oxidation (Athenstaedt & Daum, 2006). The mobilization and/or utilization of fatty acids stored in lipid droplets of the fat bodies can also be used in many insect physiological processes, including flight, synthesis of trehalose and proline, and enduring starvation (Arrese & Soulages, 2010). Accordingly, the higher triglyceride content in late season males might be associated with the longer flight distances needed to locate more than one female for mating under the more pronounced skewed sex ratio during this season.

At the biochemical level, insect resistance to xenobiotics typically involves increases in the metabolic activities of detoxification enzymes such as esterases, cytochrome P450 monooxygenases (P450s), and glutathione-*S*-transferases (GSTs) (Li *et al.*, 2007). Bark beetles have previously been found to show various responses to host tree-produced toxins related to the expression of genes coding for detoxication enzymes such as P450s, GSTs, and esterases after treatment with terpenoids and feeding with host phloem (Keeling *et al.*, 2012, 2013; Cano-Ramírez *et al.*, 2013; López *et al.*, 2013; Dai *et al.*, 2015, 2016; Chiu *et al.*, 2017). Adult beetles of different sexes and generations are assumed to differ in certain physiological and/or biochemical respects, and indeed they have been shown to have different tolerances to host monoterpenes (Reid & Purcell, 2011; Dai *et al.*, 2015; Chiu *et al.*, 2017).

Direct exposure of bark beetles to monoterpenes has indicated that individual beetles with greater amounts of stored lipid are more likely to enter media amended with monoterpenes than are those with lower lipid levels (Wallin & Raffa, 2000, 2002, 2004). Similarly, the sex-dependent survivorship of mountain pine beetles has been demonstrated to be related to differences in the body size and fatty content of males and females (Reid & Purcell, 2011). The females of Chinese white pine beetle also have larger body mass and higher fatty acid content than males in both seasons, which might lead to a higher sex-dependent survivorship (Dai *et al.*, 2015). However, we found that in both first- and second-generation adults, the content of cytochrome b5, and reduced glutathione and carboxylesterase activities, were not at higher levels in females than in males. Bark beetles have a number of detoxificative enzymes to deal with different terpenoids, with certain enzymes responding to specific monoterpenes (Cano-Ramírez *et al.*, 2013; López *et al.*, 2013; Dai *et al.*, 2015, 2016). We found that some of the P450 genes we examined were less abundant in males from both seasons (fig. 2), which could be related the biochemical differences underlying the differential survivorship of males and females.

With regards to generational differences between males and females, we noted some interesting differences in bark beetles' energy store and detoxificative enzymes. Both early season male and female beetles have higher glycogen content than late season males and females, which is presumably related to the fact that early season beetles need to endure cold temperatures and drought in spring, which are conditions that late season beetles in summer would not encounter. The higher glycogen content seen in early season beetles has similarly been noted in several other insects (Overgaard *et al.*, 2007; Vanin *et al.*, 2008), and indicates that cold acclimation leads to an increase in body content of trehalose and glucose.

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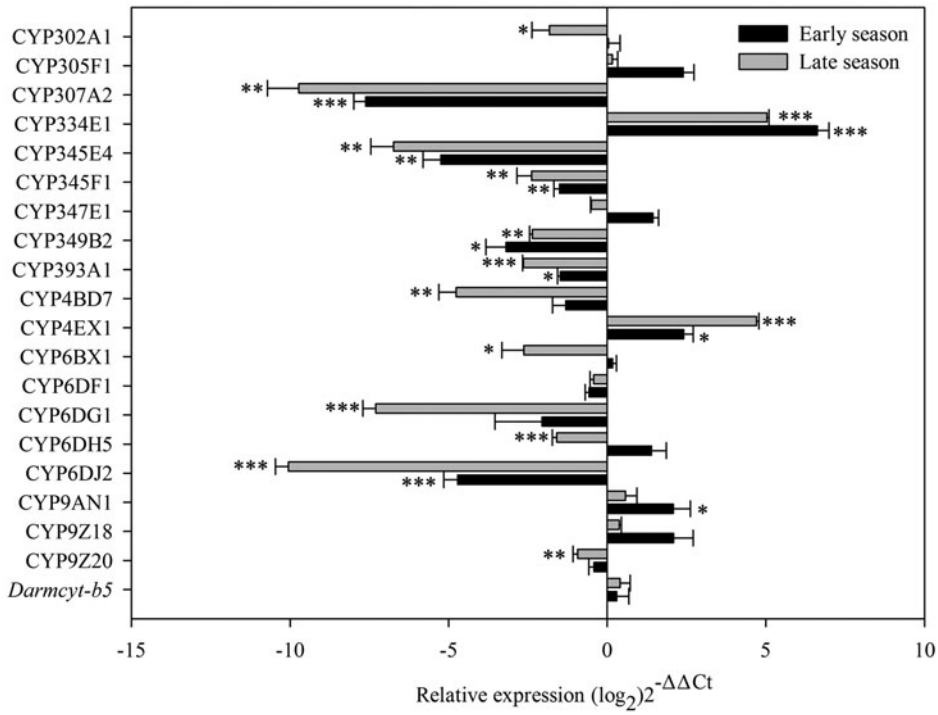

Fig. 2. Quantitative real-time PCR analysis of transcript levels of 19 cytochrome P450 (CYP) genes and *DarmCyt-b5* in males related to females from two season adults of *D. armandi* (male/female). $2^{-\Delta\Delta Ct}$ values were log2 transformed for plotting as mean ± SE. Each gene transcript level was obtained from three biological replicates (three beetles for one replicate) ($N = 3 \times 3$). * indicate the significant difference between sexes of the same season with One-way ANOVA (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Furthermore, our observations that the transcription levels of more P450 genes had significant differences between sexes in late season compared with those in early season might be indicate that the host conditions in colonizing for early season beetles are different from late season beetles. Considering the specific behavior of adult beetles, in which females are the first to arrive at the host to commence excavation through the outer bark and phloem (Li & Zhou, 1992; Latty & Reid, 2009), a skewed sex ratio in favor of females might be necessary to ensure the survival of sufficient numbers for successful colonization.

Summarizing our findings, a female-biased skewed sex ratio was found in both seasons of Chinese white pine beetles. Compared to the males, the females had a larger body mass and higher amounts of stored lipids, which are assumed to be adaptations designed to overcome host resistance and facilitate subsequent oviposition. However, further investigations are needed to examine overwinter larval mortality and oviposition characteristics in both early and late season females to elucidate the factors contributing to the regulation and stabilization of Chinese white pine beetle population size.

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