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Esterase in Imported Fire Ants, *Solenopsis invicta* and *S. richteri* (Hymenoptera: Formicidae): Activity, Kinetics and Variation

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Solenopsis invicta **and** ***Solenopsis richteri*** **are two closely related invasive ants native to South America. Despite their similarity in biology and behavior,** ***S. invicta*** **is a more successful invasive species. Toxic tolerance has been found to be important to the success of some invasive species. Esterases play a crucial role in toxic tolerance of insects. Hence, we hypothesized that the more invasive** ***S. invicta*** **would have a higher esterase activity than** ***S. richteri.*** **Esterase activities were measured for workers and male and female alates of both ant species using α-naphthyl acetate and β-naphthyl acetate as substrates. Esterase activities in** ***S. invicta*** **were always significantly higher than those in** ***S. richteri*** **supporting our hypothesis. In** ***S. invicta*****, male alates had the highest esterase activities followed by workers then female alates for both substrates. In** ***S. richetri*****, for α-naphthyl acetate, male alates had the highest activity followed by female alates then workers, while for β-naphthyl acetate, female alates had the highest activity followed by male alates then workers. For workers,** ***S. richteri*** **showed significantly higher levels of variation about the mean esterase activity than** ***S. invicta*****. However,** ***S. invicta*** **showed significantly higher levels of variation in both female and male alates.**

The red imported fire ant, *Solenopsis invicta* (Hymenoptera: Formicidae), is an infamous global invasive pest ant. Native to South America, *S. invicta* has been introduced into many countries and regions[1] and has a great potential for further spread[2]. *Solenopsis invicta* is not only a significant threat to public health due to their venomous stings, but is also an important pest in agriculture by damaging crops and interfering with farming operations. *Solenopsis invicta* causes $6 billion in estimated annual economic loss in the United States alone[3]. The ecological impact of *S. invicta* is also enormous. For instance, *S. invicta* not only reduces species density of native ants at local scales, but also changes the co-occurrence patterns of surviving ant species at a biogeographic scale[4]. Due to its great social, economic and ecologic importance, *S. invicta* has been included in the "100 of the World's Worst Invasive Alien Species"[5].

The black imported fire ant, *Solenopsis richteri*, also native to South America, is a species closely related to *S. invicta*. These two species invaded the United States through the same entrance: the port of Mobil, Alabama. Although *S. richteri* was introduced and established earlier than *S. invicta*[6], the latter has successfully displaced *S. richteri* throughout most of its distribution[6–10]. In contrast to the wide global distribution of *S. invicta*, *S. richteri* has established outside its native range only in the southern United States and currently limited to small area in north Mississippi, north Alabama and southern Tennessee. These two species are very similar in morphology, biology, colony structure, foraging and feeding behavior, and diet[11], which caused confusion in the early literature as to species identity. Hybridization between these two species occurs in the United States[12], indicating their high genetic and ecological similarity. However, the difference in the overall invasion success and their interaction in the United States indicate that *S. invicta* must possess some traits that provide a competitive advantage over *S. richteri*. *S. invicta* is a well-known disturbance specialist that thrives in the disturbed habitats[6,13]. It has been suggested that a species with higher toxic tolerance is likely to have a strong advantage in disturbed or polluted habitats[14]. Esterases plays a crucial role in toxic tolerance of insects due to their detoxification functions. A wide range of different esterases exist in insects and they differ in their substrate specificity and function[15]. Esterase isozymes in insects, particularly those in *Drosophila* and mosquitoes, are classified according to their preferential

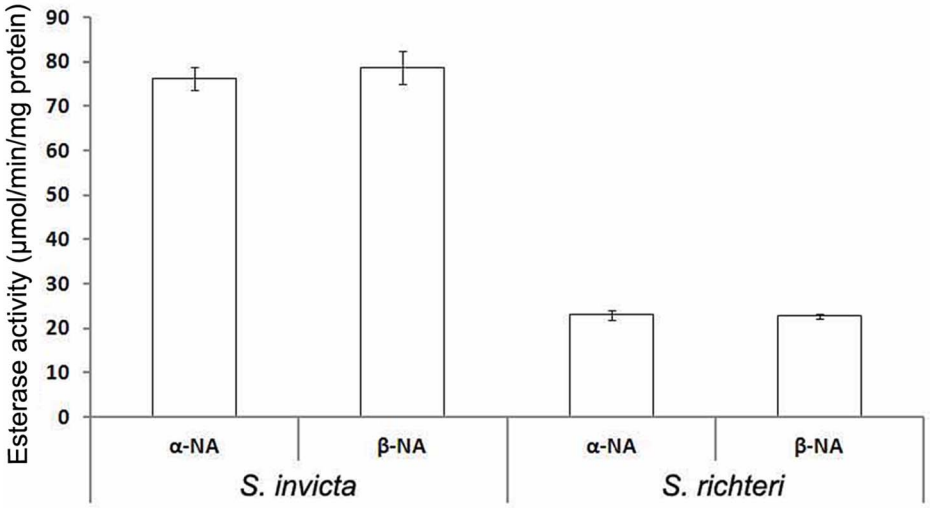

Figure 1 | Mean esterase activities (±SE) in workers of *Solenopsis invicta* and *S. richteri* for hydrolyzing α-naphthyl acetate (α-NA) and β-naphthyl acetate (β-NA), which were determined by using enzyme preparations of 5 workers. The difference between two ant species is significant for both α-NA ($t = 13.39$, $df = 67$, $P = 0.0002$) and β-NA ($z = -6.85$, $P < 0.0001$).

hydrolysis of the isomeric artificial substrates, α-naphthyl acetate (α-NA) and β-naphthyl acetate (β-NA)[15]. Although the classification has little value as a predictor of enzyme function, esterase activities measured using those substrates have often been proven to be associated with insecticide tolerance and resistance[16–26]. In this study, we tested a hypothesis that *S. invicta* has higher esterase activity than *S. richteri*. The esterase activities in workers and male and female alates of both species were analyzed using α-NA and β-NA. The kinetics of esterase was established for both species and substrates. The inhibition of esterase was also investigated using eserine, a carbamate, malaoxon, an organophosphate and 2-tridecanone, a common ant defensive compound.

Results

Esterase activity. Esterase activities determined using enzyme preparation of 5 workers are shown in Fig. 1. *Solenopsis invicta* always had significantly higher esterase activity than *S. richteri* for both α-NA ($t = 13.39$, $df = 67$, $P = 0.0002$) and β-NA ($z = -6.85$, $P < 0.0001$). For each substrate, significant difference in esterase activity was found among colonies within each species except β-NA in *S. invicta* (α-NA in *S. invicta*: $F_{2, 30} = 5.51$, $P = 0.0092$; β-NA in *S. invicta*: $H = 3.00$, $df = 2$, $P = 0.22$; α-NA in *S. richteri*: $F_{2, 33} = 8.64$, $P = 0.001$; β-NA in *S. richteri*: $F_{2, 33} = 10.10$, $P = 0.004$).

Esterase activities determined by using individual ants are summarized in Table 1. *Solenopsis invicta* always had significantly higher mean esterase activity than *S. richteri* for both α-NA (worker: $z = -11.26$, $P < 0.0001$; female alate: $z = -4.91$, $P < 0.0001$; male alate: $z = 9.07$, $P < 0.0001$) and β-NA (worker: $z = -13.29$, $P < 0.0001$; female alate: $z = -4.30$, $P < 0.0001$; male alate: $z = 11.11$, $P < 0.0001$). Pairwise comparison between castes within a species was all significant for both substrates ($P \leq 0.0009$) (Table 1S). Male alates had significantly higher esterase activity than workers for both species on both substrates. On α-NA, male alates had significantly higher esterase activity than female alates for both species. So did *S. invicta* male alates on β–NA. However, *S. richteri* female alates had higher activity than male alates on β–NA. Esterase activity in individual workers is shown in Fig. 2. For both substrates, *S. richteri* had a greater variance than *S. invicta*. The data for *S. invicta* was normally distributed for both substrates. In contrast, data for *S. richteri* were not normal for both substrates (Figure S1). Esterase activities of individual female alates are shown in Fig. 3. *S. invicta* had a greater variance than *S. richteri* for both substrates. The data for *S. richteri* was normally distributed, but not the data for *S. invicta* (Figure S2). Esterase activity in male alates is shown in Fig. 4. *S. invicta* also had a greater variance than *S. richteri* for both substrates. All data were not normally distributed except the data for *S. richteri* on β-NA (Figure S3).

Esterase kinetics. Kinetic parameters of esterases for α-NA and β-NA are shown in Table 2 and Table 3 respectively. Two types of V*max* values were calculated: one based on individual ants (μmol/min/ant) and the other on amount of protein (μmol/min/mg protein). Ratios of *K*m and *V*max values between two ant species (*S. invicta*/*S. richteri*) are shown in Table 4. For α-NA, the difference in *K*m values between two ant species was not significant ($t = -0.79$,

Table 1 | Esterase activities in individual ants of *Solenopsis invicta* and *S. richteri* for hydrolyzing α-naphthyl acetate (α-NA) and β-naphthyl acetate (β-NA). Since protein in individual ant was not determined, the activity is presented as μg/min/mg ant

		Esterase activity (mean ± SE) (μg/min/mg ant)	
Substrate	Caste	*S. invicta*	*S. richteri*
α-naphthyl acetate	Female alate	0.21 ± 0.0056 c	0.18 ± 0.0012 b
	Male alate	0.36 ± 0.0156 a	0.24 ± 0.0028 a
	Worker	0.24 ± 0.0039 b	0.15 ± 0.0053 c
β-naphthyl acetate	Female alate	0.24 ± 0.0069 c	0.21 ± 0.0022 a
	Male alate	0.32 ± 0.0126 a	0.20 ± 0.0028 b
	Worker	0.29 ± 0.0050 b	0.11 ± 0.0062 c

*: Within a species and a substrate, means followed by the different letter are significantly different ($p < 0.05$).

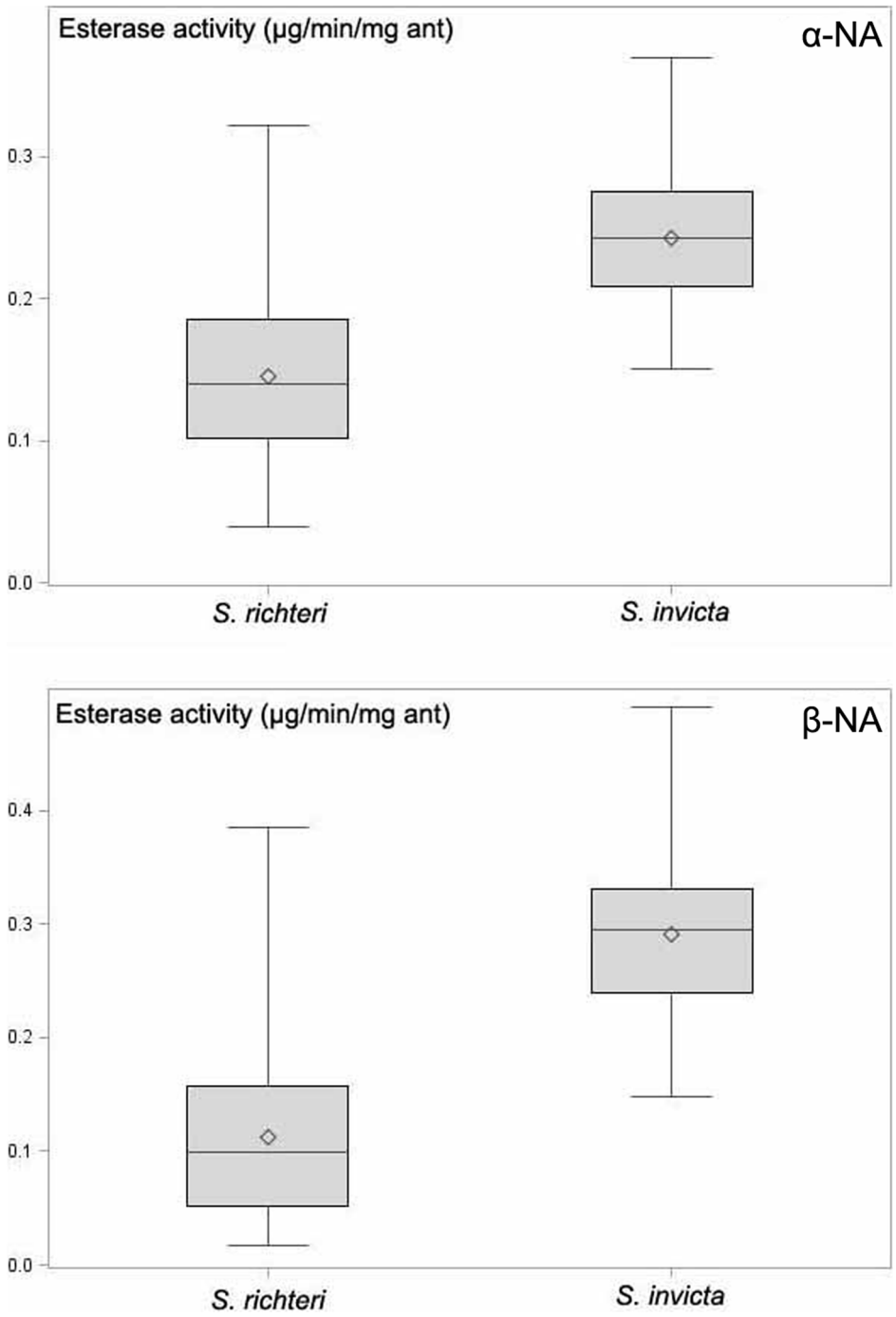

Figure 2 | **Esterase activity in individual workers of *Solenopsis invicta* and *S. richteri* based on two substrates, α-naphthyl acetate (α–NA) and β-naphthyl acetate (β-NA).**

df = 16, *P* = 0.44). The difference was significant among colonies in *S. invicta* ($F_{2,6}$ = 7.09, *P* = 0.03), but not in *S. richteri* ($F_{2,6}$ = 1.97, *P* = 0.22). *Solenopsis invicta* had significantly greater ant based *V*max (μmol/min/ant) than *S. richteri* (*t* = −5.33, *df* = 16, *P* < 0.0001). The difference among colonies was significant for both *S. invicta* ($F_{2,6}$ = 5.18, *P* = 0.049) and *S. invicta* ($F_{2,6}$ = 7.13, *P* = 0.026). *Solenopsis invicta* had greater protein based *V*max (μmol/min/mg protein) than *S. richteri* and the difference was very close to be statistically significant (*t* = −2.08, *df* = 16, *P* = 0.054). The difference among colonies was significant in *S. invicta* ($F_{2,6}$ = 10.84, *P* = 0.01) but not in *S. richteri* ($F_{2,6}$ = 3.12, *P* = 0.12). For β-NA, the difference in mean *K*m values was not significant between two ant species (*t* = −1.26, *df* = 16, *P* = 0.23). The difference was significant among colonies in *S. invicta* ($F_{2,6}$ = 6.73, *P* = 0.03), but not in *S. richteri* ($F_{2,5}$ = 5.54, *P* = 0.054). *Solenopsis invicta* had significantly greater ant based *V*max (μmol/min/ant) than *S. richteri* (*t* = −4.50, *df* = 15, *P* = 0.0004). The difference among colonies was not significant for both *S. richteri* ($F_{2,5}$ = 1.80, *P* = 0.25) and *S. invicta* ($F_{2,6}$ = 3.02, *P* = 0.12). *Solenopsis invicta* had greater protein based *V*max (μmol/min/mg protein) than *S. richteri* (*t* = −2.20, *df* = 15, *P* = 0.044). The difference among colonies was significant in *S. invicta* ($F_{2,6}$ = 7.97, *P* = 0.02) but not in *S. richteri* ($F_{2,5}$ = 2.95, *P* = 0.14).

Inhibition of esterase by 2-tridecanone, eserine and malaoxon. All three inhibitors caused a significant reduction in esterase activities in both species. Inhibition rates by 2-tridecanone and eserine are shown

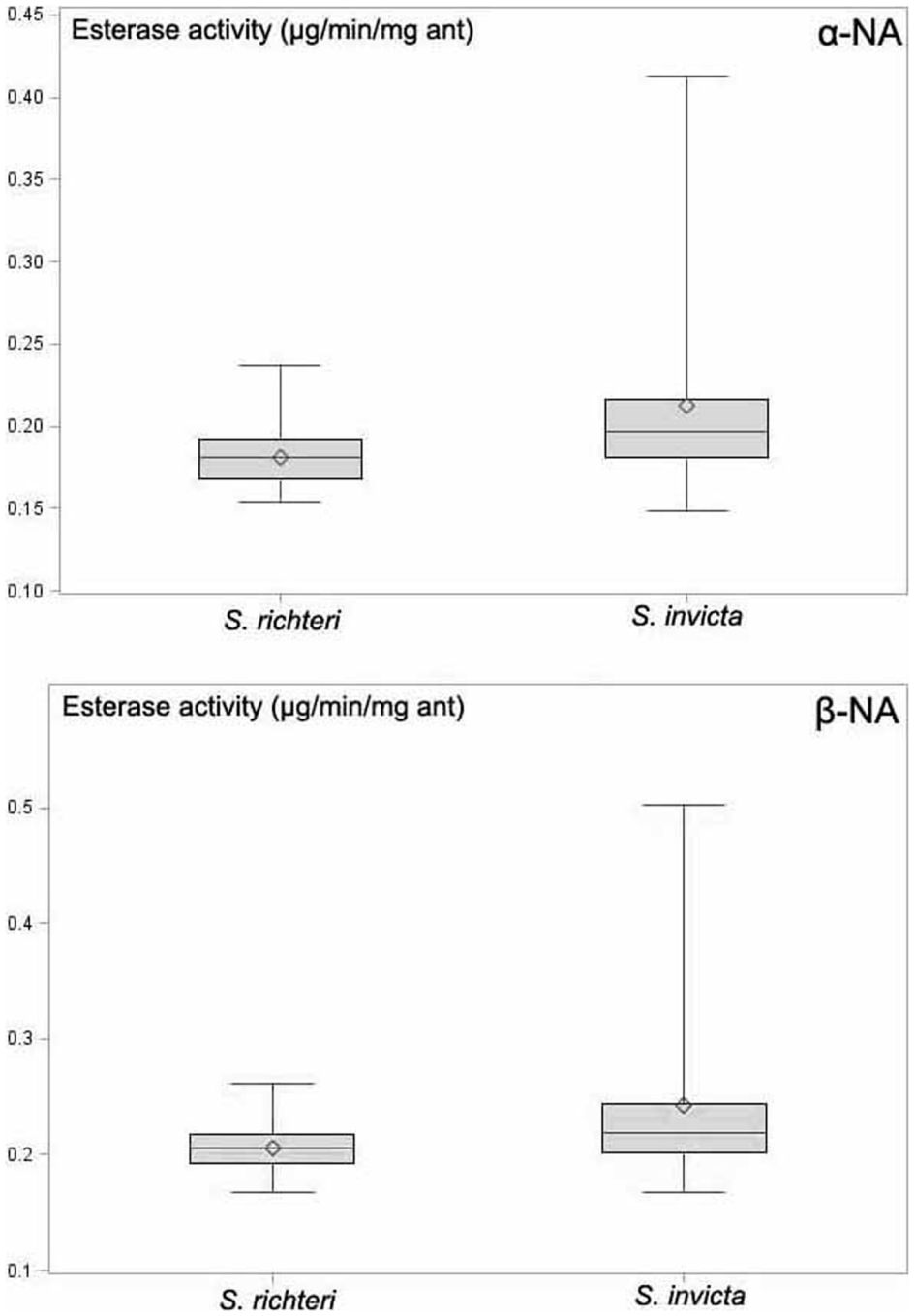

Figure 3 | **Esterase activity in individual female alates of *Solenopsis invicta* and *S. richteri* based on two substrates, α-naphthyl acetate (α-NA) and β-naphthyl acetate (β-NA).**

in Fig. 5. I_{50} values in inhibition by malaoxon are shown in Table 5. For α-NA, 2-tridecanone inhibited 6.74% and 25.90% esterase activity in *S. invicta* and *S. richteri* respectively. The difference between species was significant ($t = 13.39$, $df = 67$, $P < 0.0001$). The difference among colonies was significant for *S. richteri* ($F_{2, 33} = 12.09$, $P = 0.0001$), but not for *S. invicta* ($F_{2, 30} = 0.02$, $P = 0.98$). For β-NA, 2-tridecanone inhibited 7.34% and 15.66% esterase activity in *S. invicta* and *S. richteri* respectively. The difference was also significant between species ($t = 5.88$, $df = 67$, $P < 0.0001$). The difference among colonies was significant for *S. richteri* ($F_{2, 30} = 3.44$, $P = 0.044$) but not for *S. invicta* ($F_{2, 30} = 2.44$, $P = 0.10$).

For α-NA, eserine inhibited 63.86% and 60.40% esterase activity in *S. invicta* and *S. richteri* respectively (Fig. 5). The difference was significant between species ($t = -3.73$, $df = 67$, $P = 0.0004$). The difference among colonies was significant for both *S. invicta* ($F_{2, 30} = 5.51$, $P = 0.0092$) and *S. richteri* ($F_{2, 33} = 8.64$, $P = 0.001$). For β-NA, eserine inhibited 40.99% and 55.92% esterase activity in *S. invicta* and *S. richteri* respectively. The difference was also significant between species ($t = 15.94$, $df = 67$, $P < 0.0001$). The difference among colonies was significant for *S. richteri* ($F_{2, 33} = 10.10$, $P = 0.0004$), but not for *S. invicta* ($F_{2, 30} = 2.58$, $P = 0.092$). For *S. invicta*, the difference in inhibition rate by 2-tridecanone between two substrates was not significant ($t = 0.45$, $df = 64$, $P = 0.65$), but the difference was significant for eserine ($t = 24.15$, $df = 64$, $P < 0.0001$). For *S. richteri*, the difference in inhibition rate between two substrates was significant for both 2-tridecanone ($t = 6.78$, $df = 70$, $P < 0.0001$) and eserine ($t = 4.88$, $df = 70$, $P < 0.0001$).

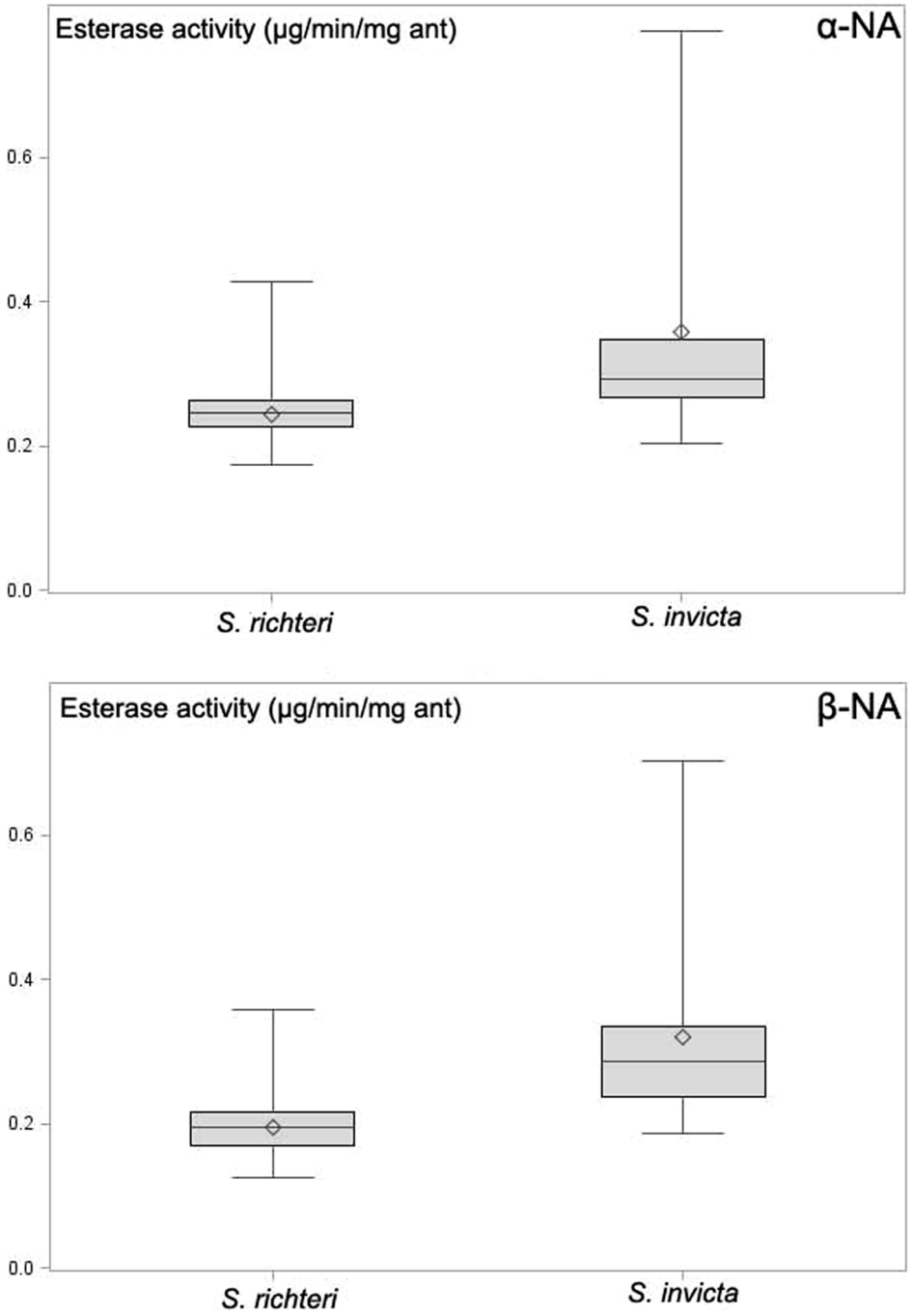

Figure 4 | **Esterase activity in individual male alates of *Solenopsis invicta* and *S. richteri* based on two substrates, α-naphthyl acetate (α–NA) and β-naphthyl acetate (β-NA).**

Table 2 | Kinetic parameters of esterases from workers of *Solenopsis invicta* and *S. richteri* on α-naphthyl acetate (α -NA)[a]

Species	Colony	Km (μM)	Vmax (μmol/min/ant)	Vmax (μmol/min/mg protein)
S. invicta	12	12.29 ± 1.68 b	12.79 ± 2.24 b	66.11 ± 9.73 b
	288	20.01 ± 1.12 a	21.15 ± 0.73 a	129.48 ± 10.05 a
	295	16.37 ± 1.02 ab	14.85 ± 1.97 ab	98.11 ± 9.33 ab
S. richteri	298	13.74 ± 0.23 a	5.94 ± 0.37 b	39.95 ± 6.38 b
	303	12.65 ± 2.20 a	4.69 ± 1.22 b	66.88 ± 3.81 ab
	6	16.81 ± 1.49 a	8.78 ± 0.47 a	85.74 ± 21.31 a

[a]The difference in *Km* values between two ant species is not significant ($t = -0.79$, $df = 16$, $P = 0.44$). *Solenopsis invicta* has significantly greater ant based *Vmax* (μmol/min/ant) than *S. richteri* ($t = -5.33$, $df = 16$, $P < 0.0001$). *Solenopsis invicta* has greater protein based *Vmax* (μmol/min/mg protein) than *S. richteri* and the difference is very close to be statistically significant ($t = -2.08$, $df = 16$, $P = 0.054$). Within a species, means followed by the different letter are significantly different ($P < 0.05$).

Table 3 | Kinetic parameters of esterases from workers of *Solenopsis invicta* and *S. richteri* on β-naphthyl acetate (β -NA)[a]

Species	Colony	Km (μM)	Vmax (μmol/min/ant)	Vmax (μmol/min/mg protein)
S. invicta	12	9.90 ± 2.04 b	11.71 ± 1.94 b	60.96 ± 9.62 b
	288	20.45 ± 2.18 a	18.59 ± 2.20 a	112.45 ± 9.68 a
	295	15.16 ± 1.85 ab	14.49 ± 1.79 ab	95.62 ± 8.12 ab
S. richteri	298	11.27 ± 0.65 ab	5.77 ± 0.34 a	39.01 ± 7.02 a
	303	7.13 ± 2.61 b	6.94 ± 2.18 a	49.97 ± 1.54 a
	6	14.38 ± 1.39 a	8.22 ± 0.62 a	78.71 ± 17.34 a

[a]The difference in mean *Km* values is not significant between two ant species ($t = -1.26$, $P = 0.23$). *Solenopsis invicta* has significantly greater ant based *Vmax* (μmol/min/ant) and protein based *Vmax* (μmol/min/mg protein) than *S. richteri* (ant based: $t = -5.08$, $df = 15$, $P = 0.0095$; protein based: $t = -2.20$, $df = 15$, $P = 0.044$). Within a species, means followed by the different letter are significantly different ($P < 0.05$).

Malaoxon inhibited esterase activity in both *S. invicta* and and *S. richteri*. For α-NA, malaoxon had greater I_{50} value for *S. richteri* than *S. invicta* ($z = 3.378$, $P = 0.0004$) and no significant difference was found among colonies for both species (*S. invicta*: $H = 2.90$, $df = 2$, $P = 0.240$; *S. richteri*: $F_{2,9} = 0.99$, $P = 0.41$). For β-NA, the difference in I_{50} value was neither significant between two ant species ($z = -0.032$, $P = 0.38$), nor among colonies (*S. invicta*: $H = 5.77$, $df = 2$, $P = 0.056$; *S. richteri*: $F_{2,9} = 1.66$, $P = 0.24$).

Discussion

This study clearly demonstrates that *S. invicta* has significantly higher esterase activity than *S. richteri*. As a critical component in insect's detoxification process, esterase has been intensively investigated in the context of insecticide resistance. Elevated esterase activities are associated with decreased insecticide susceptibility in numerous insects[16–26]. The detoxifying enzymes are important not only in tolerance and resistance of insects to both synthetic and natural toxins, but also in defense against microbial infection. For example, mosquito larvae with higher detoxification capability were more tolerant to toxicants from leaf litter[27] and to infection caused by an entomopathogenic bacteria, *Bacillus thuringiensis* var. *israelensis*[28]. Esterases in *Locusta migratoria* were involved in the defense against the infection of an entomopathogenic fungus *Metarhizium anisopliae*[29].

Having a high detoxification enzyme activity must be important to the success of ants, since they frequently encounter toxins and pathogenic microorganisms in their life. The most important natural enemies of ants are probably other ants. Many ants possess toxic venom which is utilized not only in their predation but also in their combats with other ants. In a combat, the venom is either injected, sprayed, or smeared to their enemies. Ants with higher tolerance to toxic venom may have an advantage in their competition with other ants. Many ants construct a nest that provides an ideal environment for pathogenic bacteria, fungi and other microbes to thrive. Adapting to the infection risks from those pathogenic microorganisms is believed to be one of driving forces in the evolution of the remarkable diversification of the social insects[30]. Ants with higher ability to defense against pathogenic microorganisms will definitely be more likely to survive in an unfavorable environment. The detoxification enzyme activity may be among the reasons why *S. invicta* is so successful.

2-Tridecanone is a common ant defensive chemical, which is used by many ant species, such as *Paratrechina longicornis*, *Gigantiops destructor*, and 14 species within genus *Myrmecocystus*[31–33]. It has also been found in tawny crazy ant, *Nylanderia fulva*[34]. Tawny crazy ant is the only ant that is found in the field to be able to displace *S. invicta*[35]. Naturally occurring 2-tridecanone was first identified in the wild tomato *Lycopersicon hirsutum* f. *glabratum*[36] and its insecticidal property has been demonstrated against insect and mite[37,38]. It is also a tick repellant[39]. 2-tridecanone may be a common natural toxin that both *S. invicta* and *S. richteri* often encounter. This study shows that esterase in *S. invicta* is significantly less sensitive to 2-tridecanone than *S. richteri*, indicating *S. invicta* may be less impacted by opponent's defense than *S. richteri* in competition with other ants, at least those ant species that utilize 2-tridecanone as their defensive chemical.

Based on their sensitivities to organophosphates (Ops), esterases are classified into three types: A-, B- and C-esterase[40,41]. Only B-esterases are readily inhibited by Ops. Esterases from both *S. invicta* and *S. richteri* are inhibited by malaoxon, indicating that they are most likely B-esterase. B-esterases can be further classified into carboxyesterases and cholinesterases based on their response to eserine[15,42]. Since esterases from both ant species are sensitive to eserine inhibition, cholinesterases and/or cholinesterase-like esterases may also be an important composition of esterases in both species.

Since male alates have limited exposure to the environment, it was unexpected to find that male alates in both species had the highest esterase activities when α-naphthyl acetate was used as a substrate. It indicates some unknown biological events specific to male alates require high esterase activity. In addition to detoxification, esterases play an important role in many other physiological activities in insects, such as the response to pheromone and plant volatiles[43–45]. What are the unique functions of esterases in male alates is an attractive subject for future research and it will also be interesting to see whether ants of different lineages have a similar trend.

In addition to detoxification enzymes, many other biological and/or ecological traits can contribute to the invasion success of a particular species. In plants, it has proven difficult to identify traits that consistently predict invasiveness and this may be largely because

Table 4 | Ratios of *Km* and *Vmax* values between two ant species (*S. invicta*/*S. richteri*)[a]

Substrate	Parameter	*S. invicta*	*S.richteri*	Ratio
α-NA	*Km*	15.77 ± 1.43	14.40 ± 0.99	1.1
	Vmax (μmol/min/ant)	16.03 ± 1.64	6.47 ± 9.31	2.48
	Vmax (μmol/min/mg protein)	94.34 ± 11.08	64.19 ± 0.73	1.47
β-NA	*Km*	14.58 ± 1.96	11.97 ± 0.91	1.22
	Vmax (μmol/min/ant)	14.62 ± 1.50	6.98 ± 10.62	2.1
	Vmax (μmol/min/mg protein)	85.83 ± 9.57	56.64 ± 9.05	1.52

[a]Ratio is calculated by dividing the mean parameter of *S. invicta* with that of *S. richteri*.

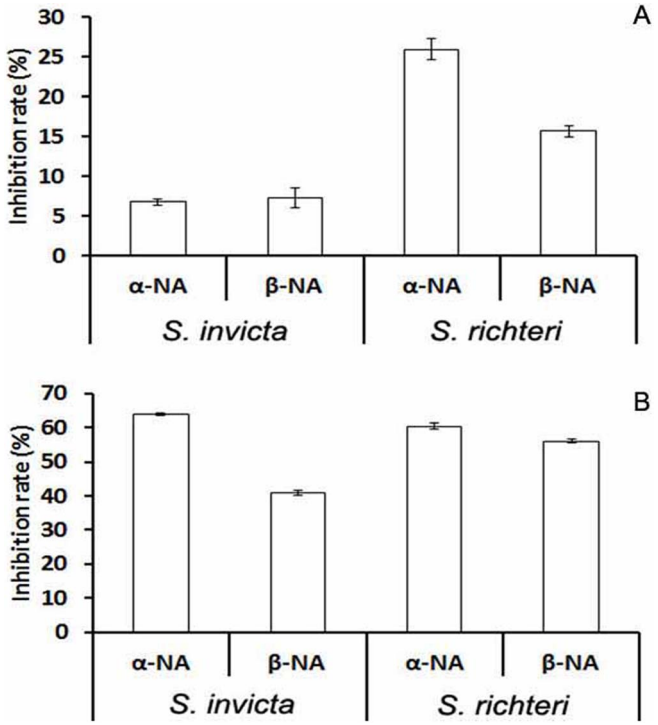

Figure 5 | Inhibition (%) of esterase activities (mean ± SD) in workers of *Solenopsis invicta* and *S. richteri* by 2-tridecanone (A) and eserine (B) on two substrates, α-naphthyl acetate (*α*-NA) and β-naphthyl acetate (β-NA).

different traits favor invasiveness in different habitats[46]. This is most likely also true to invasive insects. Whether the difference in esterase activity observed in this study is indeed associated with the variation in invasion success between *S. invicta* and *S. richteri* can only be confirmed by further comparative studies between these two species and function analysis of such differences in different habitats.

Methods

Ants. Twenty *Solenopsis invicta* colonies were collected from Washington County, Mississippi and 20 *S. richteri* colonies from Desoto County, Mississippi. Permission for collecting ant colonies on highway right-of-way was issued by Mississippi Department of Transportation. Ant colonies were maintained in an insect rearing room at 25°C. All colonies used in this study were ensured to be free of *Kneallhazia solenopsae*, a microsporidian pathogen. The social form of *S. invicta* colonies was determined using PCR on Gp-9 alleles. Method described by Valles and Porter[47] was used to amplify Gp-9 alleles. All ants used in this study were from monogyne colonies.

Table 5 | I_{50} values of malaoxon in the inhibition of esterases from workers of *Solenopsis invicta* and *S. richteri* on α-naphthyl acetate (α -NA) and β-naphthyl acetate (β -NA)

Species	Substrate	Colony	I_{50} (mM, mean ± SE)*
S. richteri	α-NA	6	0.086 ± 0.012 a
		9	0.099 ± 0.004 a
		14	0.142 ± 0.041 a
	β-NA	6	0.159 ± 0.022 a
		9	0.210 ± 0.029 a
		14	0.331 ± 0.060 a
S. invicta	α-NA	284	0.040 ± 0.011 a
		2	0.027 ± 0.008 a
		12	0.047 ± 0.005 a
	β-NA	284	0.308 ± 0.037 a
		2	0.221 ± 0.067 a
		12	0.068 ± 0.001 a

*: Within a species and substrate, means followed by the same letter are not significantly different ($P < 0.05$).

Mature queen-right colonies with brood and alates were used in the study. All colonies were on the same diet (10% sugar water and house crickets). Only newly collected ant colonies were used.

Chemicals. 2-tridecanone, Fast Blue B salt [o-Dianisidine bis(diazotized) zinc double salt; DBB], sodium dodecyl sulfate (SDS), α-naphthyl acetate (α-NA), β-naphthyl acetate (β-NA) α-naphthol, β-naphthol, malaoxon and eserine were purchased from Sigma Aldrich (St Louis, MO, USA). Stock solutions of both α-NA and β-NA were prepared in absolute ethanol.

Enzyme preparation. In addition to individual ants, the esterase activity was also determined using enzyme preparation of 5 workers. The enzyme preparations of 5 workers were also used in determining esterase kinetics and inhibition. Ant(s) was grinded using a pestle in a 1.5-ml centrifuge tube after being frozen at −80°C for 24 h. The sample was homogenized thoroughly for about 1 min at 4,000 rpm in 40 mM sodium phosphate buffer (pH 7.4) containing 0.02% Triton X-100. Five hundred microliter of sodium phosphate buffer was used for the preparation of 5 workers; 200 μl for individual worker; 400 μl for individual alate (male or female). The preparation was then centrifuged at 8,000 rpm for 10 min by using a microcentrifuge (Beckman Coulter, Fullerton, CA). The supernatant was decanted and filtered through glass wool. Before enzyme bioassay, for the preparation of 5 workers, the supernatant was diluted 10 fold for *S. invicta* and 5 fold for *S. richteri*. No dilution was made for preparations of individual ant.

Esterase activity. A colorimetric method was adapted for measuring esterase activity using α-NA or β-NA as substrates[48]. In brief, esterases hydrolyzed α- or β-naphthyl acetate into α- or β-naphthol and acetate. The amount of α- or β-naphthol produced was used to determine the esterase activity. A reaction mixture consisted of one of the following preparations in a 1.5-ml centrifuge tube:

1. 40 μl of enzyme preparation of 5 workers (0.04 and 0.08 ant equivalent for *S. invicta* and *S. richteri* respectively), 950 μl 40 mM sodium phosphate buffer (pH 7.4), and 10 μl 1×10^{-2} M α-NA or 5×10^{-3} M β-NA.
2. 10 μl of individual female alate preparation, 980 μl 40 mM sodium phosphate buffer (pH 7.4), and 10 μl 2.5×10^{-3} M α-NA or β-NA.
3. 20 μl of individual worker or male alate preparation, 970 μl 40 mM sodium phosphate buffer (pH 7.4), and 10 μl 2.5×10^{-3} M α-NA or β-NA.

The final volume of the mixture was 1.0 ml. After the mixture was incubated at 37°C for 30 minutes, 150 μl Fast Blue B-SDA–SDS solution (1.0% Fast Blue salt: 5% SDS = 2 : 5) was added to stop the reaction. Color was developed for 10 min and the absorbance was then measured using a UV-VIS spectrophotometer (Spectro UV-Vis Auto UV-2602, Labomed Culver City, CA) at λ = 600 nm for α-naphthol or λ = 560 nm for β-naphthol. The blank cuvette contained all ingredients except substrate. The concentration of α- or β-naphthol was determined from a standard curve of α- or β-naphthol. For determining esterase activities using preparations of 5 workers, 3 colonies were used for each species and there were 9–12 replicates for each colony. For individual worker, 7 colonies were used for each species and there were 20 replicates for each colony. For individual female alate, 4 *S. richteri* colonies and 5 *S. invicta* colonies were used. There were 12 to 24 replicates for each colony. For individual male alate, 8 *S. richteri* colonies and 4 *S. invicta* colonies were used. There were 15 to 35 replicates for each colony.

Determination of protein contents. Protein content in the enzyme preparation of 5 workers was determined for each enzyme preparation using the method described by Bradford[49]. Bovine serum albumin was used as the standard and absorbance was measured at λ = 595 nm. Protein content in the enzyme preparation of individual ant was not determined.

Esterase kinetics. V_{max}, the enzyme's maximum velocity, and K_m, the Michaelis constant, were determined for both α-NA and β-NA using enzyme preparations of 5 workers. Reaction mixture consisted of 50 μl diluted enzyme preparation, 940 μl 0.04 M sodium phosphate buffer (pH 7.4), and one of the following six concentrations of substrate: 2.5×10^{-3}, 5×10^{-3}, 2.5×10^{-2}, 5×10^{-2}, 2.5×10^{-1}, and 5×10^{-1} mM. Color development and absorbance measurement were the same as described above. V_{max} and K_m, were calculated using Lineweaver-Burk Plot[50]. Three colonies were used for each species. There were 2 to 4 replicates for each colony.

Inhibition of esterase by 2-tridecanone, eserine and malaoxon. The inhibition of esterase activity by 2-tridecanone, esterine and malaoxon was measured for both α- and β-naphthyl acetate using enzyme preparations of 5 workers. 2-tridecanone and eserine were tested only at 0.25 M and 5×10^{-4} M respectively; whereas 5 concentrations of malaoxon were tested, including 1×10^{-3}, 5×10^{-4}, 1×10^{-4}, 5×10^{-5}, and 1×10^{-5} M. Test compound (5 μl) was added in a 1.5 ml centrifuge tube. In control, only acetone, the solvent for all tested compounds, was added. After acetone was completely evaporated (10 min) at room temperature, 40 μl diluted enzyme preparation was added into the tube. After the mixture was pre-incubated at 37°C for 10 min, 945 μl 40 mM sodium phosphate buffer (pH 7.4), and 10 μl 2.5×10^{-3} M α-NA or β-NA were added. After the mixture was incubated at 37°C for 30 minutes, 150 μl Fast Blue B-SDA–SDS solution was added to stop the reaction. The inhibition rates (%) for 2-tridecanone and eserine and I_{50} values (concentration that causes 50% enzyme inhibition) for malaoxon were calculated. Three colonies from each species

were used for each compound. There were 9–12 replicates from each colony for inhibition rate of 2-tridecanone and serine and 3–5 replicates for I_{50} value of malaoxon.

Data analysis. Whenever data was normally distributed, a *t*-test was used for pairwise comparison in difference of esterase activity, parameter of enzyme kinetics, inhibition rate and I_{50} value between two ant species using pooled cross-colony data. Otherwise, Mann–Whitney U test was used. For comparison among colonies, analysis of variance (PROC GLM, SAS Institute 2008) was used when data was normally distributed, otherwise Kruskal-Wallis test was used.

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Author contributions

Conceived the experiments: J.C. and T.R. Designed the experiment: J.C. and G.F. Performed the experiments: G.F. Analyzed the data: J.C., G.F. and T.R. Wrote the manuscript: J.C. All authors read and approved the final manuscript.

Additional information

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