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RESEARCH ARTICLE

Food searching behaviour of a Lepidoptera pest species is modulated by the *foraging* gene polymorphism

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ABSTRACT

The extent of damage to crop plants from pest insects depends on the foraging behaviour of the insect's feeding stage. Little is known, however, about the genetic and molecular bases of foraging behaviour in phytophagous pest insects. The *foraging* gene (*for*), a candidate gene encoding a PKG-I, has an evolutionarily conserved function in feeding strategies. Until now, *for* had never been studied in Lepidoptera, which includes major pest species. The cereal stem borer *Sesamia nonagrioides* is therefore a relevant species within this order with which to study conservation of and polymorphism in the *for* gene, and its role in foraging – a behavioural trait that is directly associated with plant injuries. Full sequencing of *for* cDNA in *S. nonagrioides* revealed a high degree of conservation with other insect taxa. Activation of PKG by a cGMP analogue increased larval foraging activity, measured by how frequently larvae moved between food patches in an actimeter. We found one non-synonymous allelic variation in a natural population that defined two allelic variants. These variants presented significantly different levels of foraging activity, and the behaviour was positively correlated to gene expression levels. Our results show that *for* gene function is conserved in this species of Lepidoptera, and describe an original case of a single nucleotide polymorphism associated with foraging behaviour variation in a pest insect. By illustrating how variation in this single gene can predict phenotype, this work opens new perspectives into the evolutionary context of insect adaptation to plants, as well as pest management.

KEY WORDS: *Foraging* gene, Candidate gene, PKG, *Sesamia nonagrioides*, Pest species, Allelic variation, Foraging behaviour, Adaptation

INTRODUCTION

The level of damage inflicted on a crop by an agricultural pest species depends on the pest's ability to colonize the field area. Colonization ability, in turn, is related to pest density and to individual propensity to oviposit or feed on multiple plants within a field. Identifying molecular mechanisms involved in plant–arthropod relationships is a prerequisite for developing specific and environmentally friendly control measures (Santiago et al., 2013). It is also a major step toward understanding evolutionary mechanisms in plant–arthropod relationships. So far, most investigations in these fields have focused on sensory mechanisms involved in plant recognition (Briscoe et al., 2013; Glaser et al., 2013; Hallem et al., 2006) or on physiological mechanisms of susceptibility or resistance to plant compounds, plant varieties or insecticidal molecules (Hogenhout and Bos, 2011; Kloth et al., 2012). Genetic and molecular bases of foraging activity are particularly well studied in *Drosophila* (Sokolowski 2001) and in the honeybee (Guan et al., 2013; Page et al., 2012), but little is known in phytophagous pest insects.

Here we present the first study of the role of the *foraging* (*for*) gene in food searching behaviour in a cereal pest lepidopteran. The order Lepidoptera includes a number of major agricultural pest species. Their larvae feed on plants, and typically have a several-week development time, during which many species move from plant to plant to complete their development. *Sesamia nonagrioides* Lefèbvre (Lepidoptera, Noctuidae) is a stem borer found on a variety of monocots belonging to the Poaceae, Cyperaceae and Typhaceae families (Le Ru et al., 2006). It is also called the Mediterranean maize borer or pink stem borer, as it is the major insect pest of maize and sorghum in several Mediterranean countries (Cordero et al., 1998; Eizaguirre and Fatinou, 2012). It can also cause economic damage to cultivated cereals in sub-Saharan West Africa (Kfir et al., 2002).

In this species, caterpillars typically move from plant to plant. In humid and mild areas, often 100% of maize plants have been attacked by the time they are harvested (Cordero et al., 1998). In addition to direct plant damage, this larval behavioural activity can seriously alter maize eatability, because *Fusarium* fungi often develop in the larval tunnels (Santiago et al., 2013). These fungi produce fumonisin mycotoxins that can accumulate in the maize kernel, and cause several pathologies in humans and other mammals, including esophageal cancer under some circumstances (Bryla et al., 2013).

The gene *for*, which encodes a cGMP-dependent protein kinase (PKG I), has been described in the literature as having a conserved evolutionary relationship with food-related behaviours. This is because it is involved in modulating foraging strategies in a variety of invertebrate species (Ben-Shahar, 2005; Fitzpatrick and Sokolowski, 2004; Fitzpatrick et al., 2005; Kaun and Sokolowski, 2009; Lucas et al., 2010; Thamm and Scheiner, 2014). The phylogenetic analysis of this gene among metazoan taxa shows high conservation of its structure and suggests a strong evolutionarily conserved link between PKG I and food-related behaviours (Fitzpatrick et al., 2005). In the nematodes *Caenorhabditis elegans* (Fujiwara et al., 2002) and *Pristionchus pacificus* (Kroetz et al., 2012), and in the fruit fly *Drosophila melanogaster* (Sokolowski, 2001), induced or natural mutations of the gene, respectively, are associated with the expression of different behavioural and physiological phenotypes. In *D. melanogaster*, flies and larvae of

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List of symbols and abbreviations

8-Br-cGMP	8-bromo-cyclic guanosine monophosphate
ARMS	amplification refractory mutation system
C_q	quantification cycle
for	*foraging* gene
Gly	glycine allele
Het	heterozygote
PKG	cGMP-dependent protein kinase
Ser	serine allele

the 'rover' genotype move farther than do individuals with the 'sitter' genotype when they forage. When food is scarce, rover larvae have higher survivorship and faster development than sitters, which is explained by their higher glucose absorption efficiency (Kaun et al., 2007). In other species, and more particularly in eusocial hymenoptera such as honeybees, *Apis mellifera* (Ben-Shahar, 2005; Thamm and Scheiner, 2014), and the ants *Pheidole pallidula* (Lucas and Sokolowski, 2009) and *Pogonomyrmex barbatus* (Ingram et al., 2005), modulation of food searching behaviours results from variation in *for* activity throughout the development of behavioural tasks. In the wasp *Vespula vulgaris* (Tobback et al., 2008) and in two *Bombus* species [*B. ignites* (Kodaira et al., 2009) and *B. terrestris* (Tobback et al., 2011)], results support the idea that *for* expression levels are related to behavioral caste differentiation and thus to *for* activity. In the locust *Schistocerca gregaria*, higher expression of the *for* orthologue characterizes the gregarious phase that typically precedes group migration (Lucas et al., 2010), and transcript levels change when the availability of food is altered and in crowd- versus isolated-reared insects (Tobback et al., 2013). In the aphid, change in *for* expression has also been observed in response to crowding, usually followed by dispersion (Tarès et al., 2013).

Sesamia nonagrioides is a relevant candidate with which to study conservation of and polymorphism in the *for* gene in a lepidopteran, and its role in a behavioural trait directly associated with plant injuries. We conducted this study with an integrative experimental approach that included analyses of sequence conservation and polymorphism, allelic frequencies, and the link between foraging activity, observed in an adapted actimeter (Chardonnet et al., 2012), and pharmacological activation of PKG, allelic variation and gene expression. By linking phenotype to genotype, this study shows how the *for* gene can modulate a behavioural function linked to pest character in a lepidopteran species.

RESULTS

The gene is well conserved in *S. nonagrioides*

We documented sequence conservation and polymorphism in *S. nonagrioides*. Primers based on *for* sequences of other lepidopterans allowed us to sequence the full-length transcript in *S. nonagrioides*. The gene cDNA was 2235 bp long, encoding 745 amino acids. We did not find evidence of several transcripts. We compared the protein sequence with the homologous *Drosophila* sequence for which the protein domains are mapped (Fig. 1). The gene was thus identified as an orthologue of the *Drosophila dg2* gene (i.e. *foraging*) coding for PKG I (Osborne et al., 1997). We labelled the gene *Snfor*.

The secondary structure of the protein was similar to that described in other species (e.g. Osborne et al., 2011). The regulatory domain is encoded by the first 185 amino acid residues, followed by cGMP1 (186–302 residues) and cGMP2 (303–418 residues) binding domains. The catalytic domain is located between the 434 and 693 residues.

A phylogenetic tree was constructed to check the position of the *S. nonagrioides* PKGI protein among other species' orthologues. It was based on 34 PKG I sequences (Fig. 2), which confirms the different clades corresponding to insect orders already evidenced by Lucas et al. (Lucas et al., 2010), and places the *S. nonagrioides* sequences in a new sub-clade gathering noctuid species within the well-supported Lepidoptera clade.

By comparing the *Snfor* protein sequence with *for* sequences from other lepidopteran species (*Bombyx mori*, AF465600; *Lobesia botrana*, DQ666642; *Sesamia calamistis*, obtained at our laboratory), we showed a high genetic identity to other orthologous genes in this order (≥94%).

The *Snfor* gene presents two allelic variants in the studied population

We compared all *Snfor* sequences obtained from cDNA of 15 individuals from a maize field collection in Haute-Garonne (France). There were several synonymous single-nucleotide polymorphisms (SNPs). We found only one non-synonymous SNP in the complete *Snfor* gene sequence. This mutation was found at the 1699 base position: an A/G nucleotide change that induces a codon change. When adenine is at the first position, the codon codes for serine in the protein sequence; when guanosine is in the first position, it codes for glycine. Mapping with the *Drosophila* sequence indicated that this substitution was located in the catalytic domain of the protein (Fig. 1). By comparison with *for* sequences from other lepidopteran species, it appeared that these other sequences had a serine at the polymorphic *Snfor* position. The GenBank accession numbers of the two variants are KC310497 ($SnFor^{Gly}$) and KC310498 ($SnFor^{Ser}$).

The next step was to quantify allelic and genotypic frequencies in a natural population from maize fields (Haute-Garonne, France). We genotyped 143 field-collected insects using the amplification refractory mutation system (ARMS) technique (Newton et al., 1989). In the samples collected yearly from 2012 to 2014 (N=94, 49 and 93, respectively), approximately 40% were $SnFor^{Gly/Gly}$ homozygotes (38 to 39%), 40–50% were $SnFor^{Gly/Ser}$ heterozygotes (51, 43 and 40%, respectively) and less than 20% were $SnFor^{Ser/Ser}$ homozygotes (10, 18 and 19%, respectively) There was no significant difference in the genotypic frequencies between the three years (Chi-square test, d.f.=2, P>0.05).

Pharmacological activation of the cGMP-dependent protein kinase increases foraging activity

To test the role of the *Snfor* gene on foraging activity in *S. nonagrioides*, and establish a causal link between the gene and the behaviour, we used a pharmacological approach that allowed us to artificially manipulate the activity level of cGMP-dependent protein kinase. It is a common technique to use a pharmacological agent to modulate specific characters, especially in non-model species where transgenic studies are not yet possible. In the literature, 8-bromo-cyclic guanosine monophosphate (8-Br-cGMP; an analogue of endogenous cGMP, the second messenger of the protein) has been described as a specific activator of PKG of *D. melanogaster* (Dawson-Scully et al., 2010), *A. mellifera* (Ben-Shahar et al., 2002) and *P. pallidula* (Lucas and Sokolowski, 2009). Here, we gave larvae a non-invasive oral chronic treatment of the PKG activator.

At the end of the treatment, they were individually tested in the 'actimeter' (Fig. 3). The 'actimeter' system was designed by Chardonnet et al. (Chardonnet et al., 2012) from a device initially developed for the fruit fly, the DAM system (see Materials and methods). This original tubular system allowed us to automatically record the number of times a larva switched between two food sources (patches).

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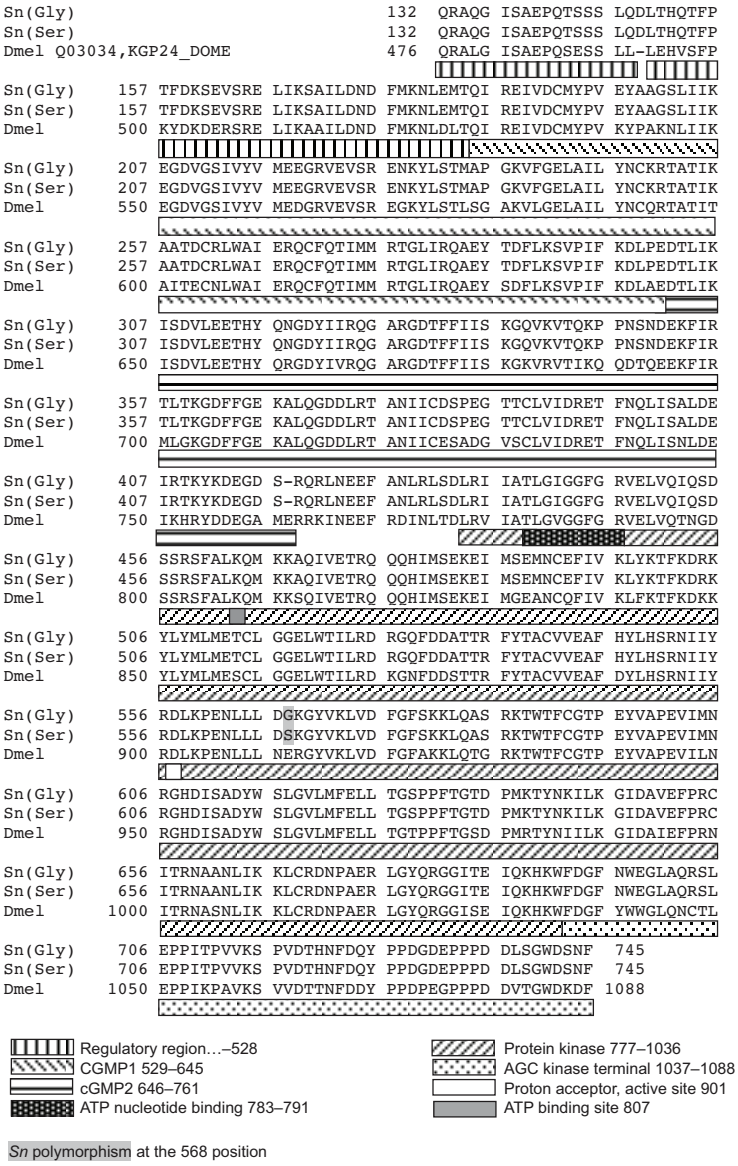

Fig. 1. Alignments of amino acid sequences of *Sesamia nonagrioides* and *Drosophila melanogaster* using Bioedit and BLAST (Kim et al., 2011; Osborne et al., 2011). Because the beginning of the *S. nonagrioides* sequence is too divergent from that of *D. melanogaster*, the alignment shown here starts at the 132th position for *S. nonagrioides* and the 449th for *D. melanogaster* (isoform A of *Dmelfor* transcript RA). The different domains of the PKG are shown for *D. melanogaster* using the UniProtKB database. The amino-acid polymorphism in *S. nonagrioides* is indicated in grey at the 567th position. From this mapping, we were able to identify the *Snfor* gene as an orthologue of the *Drosophila dg2* gene (i.e. *foraging*) coding for the PKG I (Osborne et al., 2011).

Foraging activity in the actimeter was significantly influenced by treatment over the first day of recording (ANOVA, $F_{3,124}$=5.138, P=0.002; Fig. 4A). The number of patch changes was higher in the group treated at 500 µmol l^{-1} (multiple comparison test, 500 versus 1000 µmol l^{-1}, P=0.018, 500 versus 0 µmol l^{-1}, P=0.012; Fisher test, P>0.05). A smaller activation was seen in the 250 µmol l^{-1} group. There was no effect at 1000 µmol l^{-1}, potentially because of toxic effects of the treatment under higher-concentration conditions (Ben-Shahar et al., 2002). We saw no effect of the treatment on the second day, suggesting a rapid decrease in efficiency of the drug once it is no longer being administered (ANOVA, $F_{3,124}$=1.011, P>0.05). The treatment had no effect on larval growth (each caterpillar was weighed each day during the treatment and at the end of the behavioural test), excluding an indirect size-related effect on behaviour. Together these data show that PKG activation increases the foraging activity observed in *S. nonagrioides* larvae.

Although PKGs are the principal target of cGMP (Hofmann et al., 2009), the great similarity between PKA (cAMP-dependent protein kinase) and PKG makes it possible to also activate PKA by cGMP, particularly when cGMP is present in high concentrations (Hofmann et al., 2009; Pilz and Castell, 2003). We therefore checked whether PKA activation influenced the foraging behaviour of *S. nonagrioides*. We substituted 8-Br-cGMP with 8-Br-cAMP, an activator of PKA (Ben-Shahar et al., 2003), using the same protocol of non-invasive oral chronic treatment (n=40 per concentration tested). At the end of the treatment, we tested larvae individually in the actimeter. The foraging activity was not significantly influenced by the treatment over the whole experiment (ANOVA, day 1: $F_{3,158}$=0.348, P>0.05; day 2: $F_{3,158}$=0.582, P>0.05; Fig. 4B). The treatment had no effect on larval size, excluding an indirect size-related effect on behaviour. These results show that PKA activation does not significantly modulate the foraging activity of *S. nonagrioides* larvae in the actimeter.

Snfor gene polymorphism is associated with the expression of different foraging phenotypes

The presence of an *Snfor* polymorphism in the studied population allowed us to test the correlation between genotypes and foraging phenotypes. We thus tested the foraging activity of larvae and genotyped them afterward.

One-hundred and seventy-nine third-instar larvae were tested in the actimeter for 3 days (72 h). We identified 59 *SnFor*$^{Gly/Gly}$

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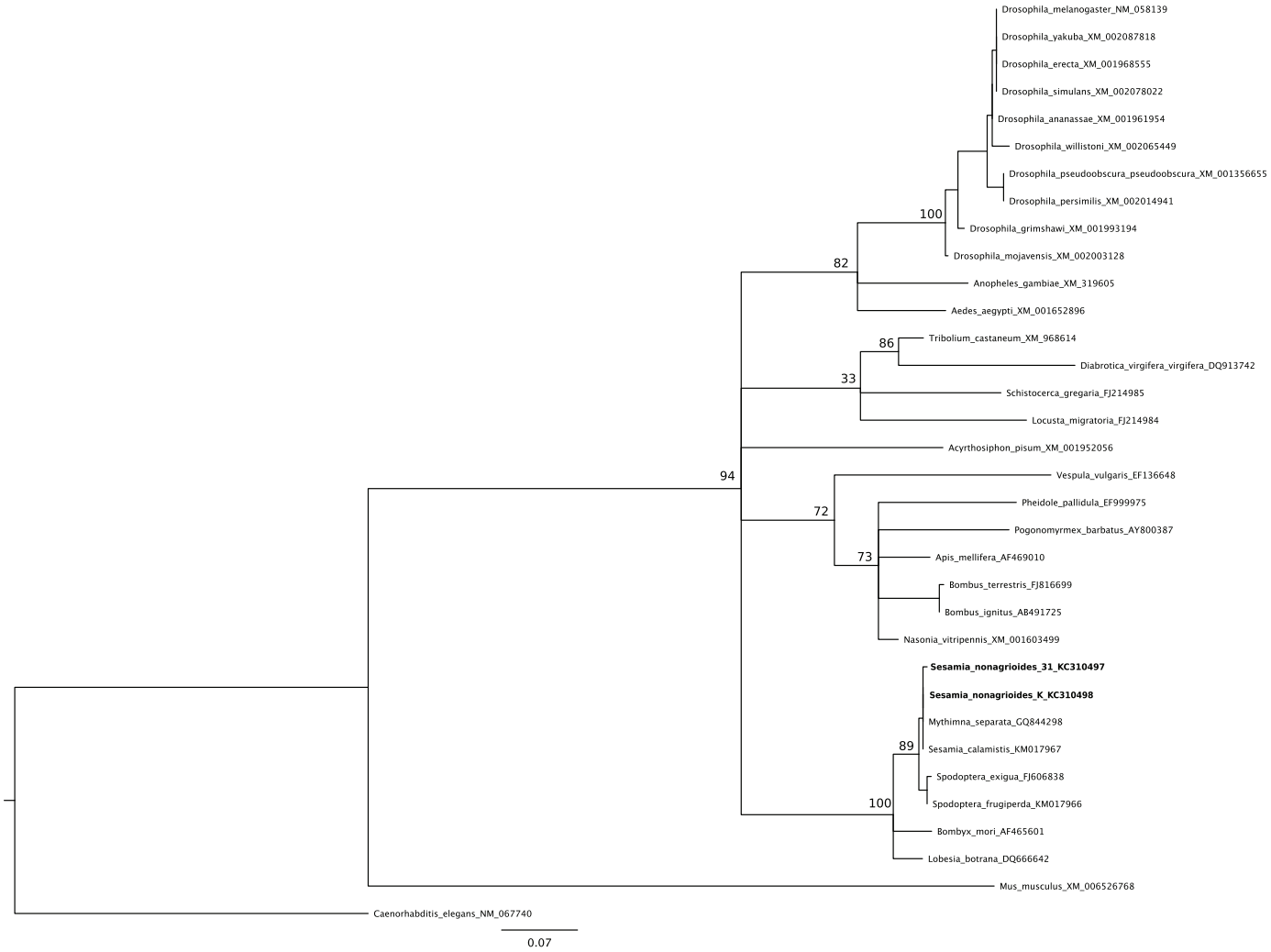

Fig. 2. Maximum likelihood phylogeny of the PKG I protein in 31 insect species. Five hundred bootstrap replications were run to check the consistency of the nodes. The two *S. nonagrioides* populations are in bold. GenBank accession numbers are given for each sequence after the name of the species. Scale of branch length is expressed in amino acid substitution rate.

homozygotes, 46 $SnFor^{Ser/Ser}$ homozygotes and 74 $SnFor^{Gly/Ser}$ heterozygotes in this laboratory-reared sample. We observed a significant effect of genotype on foraging activity (Kruskal–Wallis test, K=13.387, d.f.=2, P=0.001; Fig. 5), revealing that $SnFor^{Ser/Ser}$ larvae left a food patch more frequently than $SnFor^{Gly/Gly}$ individuals, while $SnFor^{Gly/Ser}$ individuals were intermediate between the two homozygotes (*post hoc* Steel–Dwass–Critchlow–Fligner test: Ser versus Gly, P=0.001; Ser versus Het and Het versus Gly, P>0.05).

These allelic frequencies were not significantly different from those observed in the analysis of field-collected larvae (Chi-square test, d.f.=4, P>0.05).

Allelic variants have different levels of *Snfor* gene expression

Variation in the level of *for* expression is known to have a major effect on the behaviour of several insect species. In social Hymenoptera, changes in the expression rates accompany the shift between nursing and foraging behaviours (Ben-Shahar et al., 2003; Heylen et al., 2008; Ingram et al., 2005; Ingram et al., 2011; Tobback et al., 2011), and constitute one of the explicative elements of the division of labour within the colony. To generate a wider view of a potential link between the behavioural phenotypes and the *Snfor* gene, we compared the gene expression level in the whole body and in the brain of the $SnFor^{Ser/Ser}$ and $SnFor^{Gly/Gly}$ homozygote strains founded from the polymorphic study population.

We first compared *Snfor* whole-body expression between $SnFor^{Ser/Ser}$ and $SnFor^{Gly/Gly}$ larvae. Individuals were taken from the two reared strains without activity testing, to avoid a situation where variation in the gene expression would result from behavioural testing (we chose the fourth-instar because larvae usually reached that stage during actimeter experiments). *Snfor* expression was significantly higher in the whole body of $SnFor^{Ser/Ser}$ larvae than in $SnFor^{Gly/Gly}$ larvae (n=30 per strain, P<0.001; Fig. 6A) (Pfaffl, 2001).

We then compared the *Snfor* brain expression between the $SnFor^{Ser/Ser}$ and $SnFor^{Gly/Gly}$ homozygote strains of *S. nonagrioides*, as above. *Snfor* expression was also higher in the brain of $SnFor^{Ser/Ser}$ larvae than in $SnFor^{Gly/Gly}$ larvae (eight pools of eight brains per population, P<0.01; Fig. 6B).

DISCUSSION

The results of these molecular, pharmacological and behavioural analyses show that the *for* gene is directly involved in modulating a

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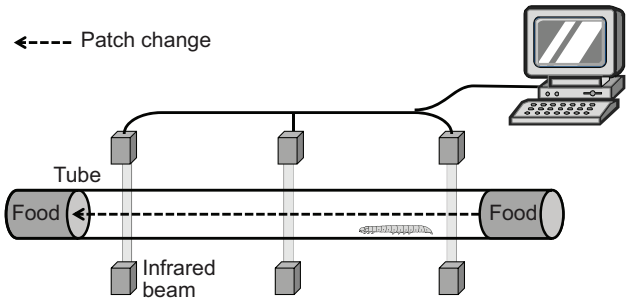

Fig. 3. Schematic illustration of one tubular unit of the actimeter, used to automatically record individual larval foraging behaviour. Displacement of each larva between distant identical food patches was recorded by three infrared beams situated along the tube (200×7 mm i.d.) (Chardonnet et al., 2012). A 'leaving the patch' event was counted when at least two successive beams were crossed, at which point most larvae walked to the next patch (82% patch change) while some returned to the initial patch. Both behaviours were pooled for the analysis of foraging activity.

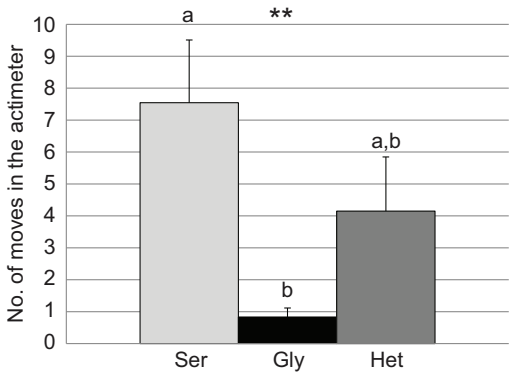

Fig. 5. Effect of the *foraging* genotype on foraging activity of third-instar larvae. Three-day recording of the number of times a larva moved between two food sources in the actimeter. **P<0.01, Kruskal–Wallis, followed by a *post hoc* Steel–Dwass–Critchlow–Fligner test (different letters indicate a significant difference at P<0.05). Ser: $SnFor^{Ser}$ (n=46); Gly: $SnFor^{Gly}$ (n=59); Het: $SnFor^{Ser/Gly}$ (n=74).

food searching trait in *S. nonagrioides*, a non-model species and an important pest of cultivated cereals in Europe.

This work showed clear sequence and function conservation of the *for* gene in *S. nonagrioides*. We concluded that *Snfor* was an orthologue of the homologous *Drosophila dg2* gene (i.e. *for*) coding for the PKG I (Osborne et al., 1997), which corresponds to the cGK I of mammals, encoded by the *prkg-1* gene (Hofmann et al., 2009). We based this conclusion on the strong conservation of sequence of the whole coding region and of the domain structure of the protein. The *Snfor* gene also has a high genetic identity to the other orthologous genes sequenced in a few Lepidoptera genera, such as *Bombyx*, *Lobesia* and *Sesamia*.

Next, by modulating foraging behaviour induced by artificially manipulating PKG activity, we identified a causal link between the PKG enzyme and the behaviour. PKG I, the *for*-encoded kinase, was very likely activated because we also showed consistent positive correlation between *Snfor* expression and foraging activity, assuming that gene expression correlates with the enzyme activity. These findings showed that the foraging function of the *for* gene is also conserved in a lepidopteran species.

In *S. nonagrioides*, we found that higher expression of *Snfor* in the brain was correlated with higher frequency of patch changes in the actimeter. There are indications in the literature of some downstream effectors, signalling pathways and sensory effects that begin to explain how PKG I activity may influence foraging traits. In the nematode *C. elegans*, EGL-4, the orthologue of *for*, has been reported to modulate gene expression of two chemosensory receptors (van der Linden et al., 2008). EGL-4 nuclear localization and activity in olfactory neurons was necessary to promote plasticity in odour responsiveness (He and O'Halloran, 2013; Juang et al., 2013). In mammals, PKG I is present in numerous tissues, and it phosphorylates more than 10 substrates known to modulate neuronal signalling pathways, including ion channels and G-protein (Hofmann et al., 2009). One of these substrates is the transporter of the neurohormone serotonin (5-hydroxytryptamine) (Zhang et al., 2007), which is involved in the regulation of feeding and digestion in many animals from worms to mammals (Gillette, 2006), including insects such as ants (Falibene et al., 2012), the honeybee (French et al., 2014) and *D. melanogaster* (Neckameyer and Bhatt, 2012). It is thus possible that variation in the *for* gene in *S. nonagrioides* may affect larval foraging behaviour via a serotonergic pathway.

One highlight of the present paper is the discovery of an SNP that correlates with differential *Snfor* expression and with differential behavioural activity. The SNP may itself have an influence on PKG activity because it is located in the catalytic site of the kinase, and may therefore affect its affinity for particular substrates. Functional consequences of single nucleotide changes have already been reported (Lenormand et al., 1999; Rottschaefer et al., 2011). It might be possible to predict the effect of the amino acid change after the 3D structure of this part of the protein is described (Osborne et al., 2011). However, a linkage disequilibrium between the SNP and possible mutations in the regulatory sequences cannot be discarded.

Our findings in *S. nonagrioides* present analogies with *D. melanogaster*. In this fly species, the *for* gene has two natural alleles that are associated with two specific foraging strategies (phenotypes) (Sokolowski 2001): rovers and sitters. Increased gene expression or PKG activity is correlated with increased foraging behaviour. Individuals of the genotype $SnFor^{Ser/Ser}$, which have a greater propensity to leave a food patch and a higher level of gene expression, could then be seen as 'rover-like', while $SnFor^{Gly/Gly}$, which are more sedentary and have a lower level of gene expression, would be 'sitter-like'. However, in *Drosophila* there are several non-synonymous mutations between the two variants and these are

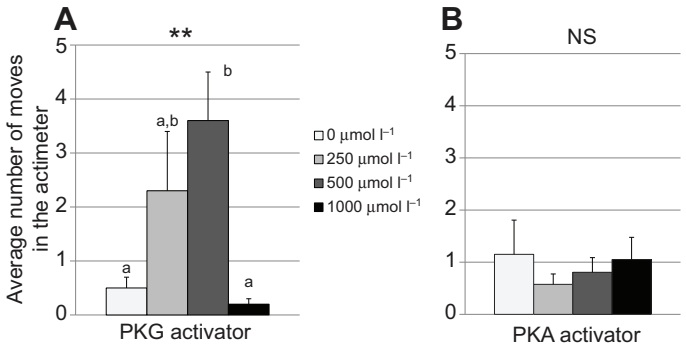

Fig. 4. Effect of pharmacological treatment on the number of patch changes. (A) Specific PKG activator on the tendency to leave a food patch in the third-instar larvae. **P<0.01, ANOVA, followed by a *post hoc* Fisher's test (different letters indicate a significant difference at P<0.05); n=40, 24, 40 and 24 for 0, 250, 500 and 1000 µmol l^{-1}, respectively. (B) Specific PKA activator on the tendency to leave a food patch in the third-instar larvae. ANOVA, P>0.5; n=40 for each tested concentration. Data are means ± s.e.m.

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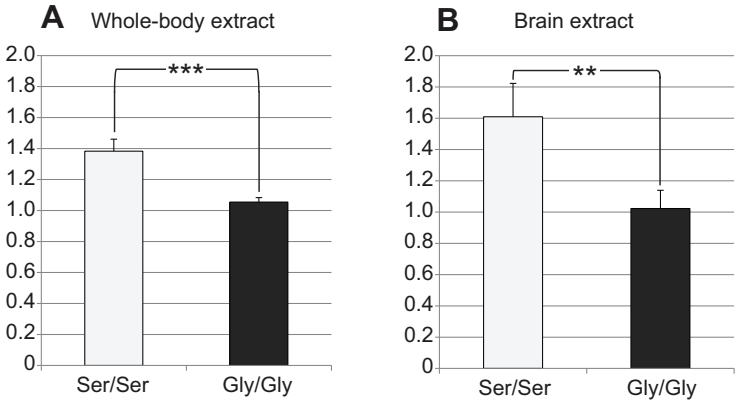

Fig. 6. Relative gene expression in the whole body and brain between the *SnFor*$^{Ser/Ser}$ and *SnFor*$^{Gly/Gly}$ homozygote strains of *S. nonagrioides*. (A) *Snfor* expression was higher in the whole body of *SnFor*$^{Ser/Ser}$ larvae than in *SnFor*$^{Gly/Gly}$ larvae. *SnFor*$^{Ser/Ser}$=1.38±0.08 and *SnFor*$^{Gly/Gly}$=1.06±0.03; $^{**}P$<0.001, n=30 larvae per strain. (B) *Snfor* expression was higher in the brain of *SnFor*$^{Ser/Ser}$ larvae than in *SnFor*$^{Gly/Gly}$ larvae. *SnFor*$^{Ser/Ser}$=1.99±0.24 and *SnFor*$^{Gly/Gly}$=1.09±0.17; $^{**}P$<0.01, n=64 larvae pooled in eight pools of eight brains per strain.

present on several transcripts of *Dmelfor* (GenBank accession numbers FJ200471–FJ200478), each coding for a different PKG I isoform that may have specific substrate. This may explain why so many functions (sensory traits, locomotion, learning traits, glucose homeostasis, etc.) differ between rovers and sitters. Our results did not reveal more than one transcript, but more specific experimental approaches (northern blot) are still needed to confirm this point.

Our *S. nonagrioides* study population was composed of the three genotypes *SnFor*$^{Gly/Gly}$, *SnFor*$^{Ser/Ser}$ and *SnFor*$^{Gly/Ser}$, with a higher frequency of the *SnFor*^{Gly} allele. In this population collected in a maize field in Haute-Garonne, the non-significant variation of the genotype ratio between larvae collected from 2012 to 2014 in the same area suggests that there is a mechanism that maintains the polymorphism. There are several possible mechanisms of polymorphism maintenance in natural populations. For example, polymorphism can be understood as a mechanism that allows species to maintain their fitness in a changing environment, as was described for the *for* gene polymorphism in the *Drosophila* model (Sokolowski et al., 1997). When food is scarce, rover flies and larvae have an adaptive advantage because they are better at finding new food patches and they assimilate nutrients more efficiently (Kaun et al., 2007). However, the high energetic cost of the rover strategy means that sitters – which use less energy for foraging – have higher fitness when food is abundant, thus maintaining a natural polymorphism (Kent et al., 2009; Sokolowski et al., 1997). This explains how the fitness of each genotype can change depending on environmental factors [gene–environment interaction (Burns et al., 2012)].

PKG I has pleiotropic effects and is also known to influence food odour learning (Mery et al., 2007) and olfactory and taste sensitivity – key components in evaluating food quality (Scheiner et al., 2004; Juang et al., 2013). As *S. nonagrioides* is able to feed and develop on several wild and cultivated host plants, our results open up new research avenues to understand some of the molecular mechanisms that contribute to the adaptation of this species to plant resources or other environmental components. The polymorphism shown here may also have an effect on the pest character of the insect, because more active variants are expected to attack more plants and favour the transmission of plant pathogens. In this context, analysis of *for* genotype frequencies in natural populations of *S. nonagrioides*, in the whole area of its distribution and on its different host plant species, opens new perspectives for predicting its damage levels in cereal crops, and for developing specific and environmentally friendly control measures.

To conclude, here we have shown conservation of *Snfor for* orthologue function for the first time in a lepidopteran, and discovered a polymorphism of the *Snfor* orthologue as a genetic mechanism that may play a role in the expression of specific foraging strategies. We illustrated how variation in this single gene could predict phenotype and discussed potential issues in the context of insect adaptation to plants and of pest management.

MATERIALS AND METHODS

Insect populations and rearing

The studied population was initiated from larvae collected from maize stems in fields located in a 10 km^2 area in Haute-Garonne, France. GPS coordinates of the central point of the collection area are 43°22.171′N, 1°11.413′E. Approximately 300 larvae were collected yearly and added to the laboratory population to maintain genetic diversity and ensure that insects were as similar as possible to those from the field. Insects were reared on an artificial diet for one to four generations before being included in the experiments, at the Laboratory Evolution, Génomes et Spéciation (LEGS, France), in incubators (26°C, 70% relative humidity, and a 16 h:8 h light:dark cycle). The diet was composed of dried maize leaf powder (13.2% dry matter), soybean powder (45.9% dry matter), saccharose (18.2% dry matter), dead pulverulent baker's yeast (11.9% dry matter), and a mix of vitamins, antibiotics, antifungal agents and preservatives (4.3% dry matter) (adapted from Onyango and Ochieng'-Odero, 1994).

Sequencing the *Snfor* gene

In order to sequence the orthologue *Snfor*, total RNA of *S. nonagrioides* was extracted from whole larvae with TRI Reagent (Sigma-Aldrich, St Louis, MO, USA). Reverse transcription was performed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Primers were designed based on *for* sequences of other Lepidoptera [*B. mori*, AF465600; *L. botrana*, DQ666642; *Mythimna separata*, GQ844298; *Spodoptera littoralis*, Poivet et al. (Poivet et al., 2013)]. These primers are D14B (ACTCTCAG-TGCTCGGTAGCC), D2 (GGCAATCYTGTACAACTSCAAGCG), R1 (GGCCCAWAGYCKGCAGTCMG), R3 (GCGCTAGRCCTTCCCAGT-TRAARCC) and R13 (AWSACWCCGAWMAMCAMT). D14B was designed in the 5′ UTR region and R13 in the 3′ UTR region. These primers allowed us to sequence the full-length transcript in *S. nonagrioides* (Fig. 1).

Phylogenetic analysis of PKG I

In order to construct the phylogeny, we used the same PKG I sequences as Lucas et al. (Lucas et al., 2010, as well as sequences of several other species: *Sesamia calamistis* (KM017967), *Spodoptera exigua* (FJ606838), *Spodoptera frugiperda* (KM017966), *Mus musculus* (XM_006526768) and *C. elegans* (NM_067740). In total, 34 sequences were used for the tree. As the N-terminal domain is very divergent and cannot be aligned across all species, only the last 323 amino acids of the C-terminal domain of the protein were kept for the phylogeny. We used the website www.phylogeny.fr (Dereeper et al., 2008, Dereeper et al., 2010) to construct our phylogeny. The sequences were first aligned with MUSCLE 3.7 (Edgar, 2004) and then a maximum likelihood tree was constructed with PhyML 3.0 (Guindon et al., 2010). Five hundred bootstrap replications were run to check the consistency of the nodes.

Analysis of the *Snfor* sequence

Once the complete cDNA sequence of the *Snfor* gene had been obtained, new primers were designed in order to sequence the whole gene in 15 individuals, directly from field collections. cDNA from each individual was amplified using three different primer combinations: D14B (see above)/R11 (GAAGAAATCGCCTTTTGTGAG); D10 (CAAGCCGAATACACCGACT)/R10 (TCCGGTAAGCAACTCAAACA) and D12 (GGGATCTCAAACCTGAGAACTT)/R15 (AATTACAAATGGCGTTAAATCT). Verification by agarose gel electrophoresis showed that only one product was amplified by each primer pair. The sequences were clean and did not show the presence of multiple transcripts.

Determination of the frequency of the two *Snfor* alleles and their related genotypes

To detect heterozygotes and to target the observed SNP, large-scale genotyping of field-collected individuals was performed on DNA using the ARMS technique (Kim et al., 2011). Genomic DNA was extracted from larvae or from adult thoraxes using a DNeasy Blood and Tissue Kit (Qiagen, Venlo, The Netherlands). Two PCRs were performed on each individual. Each PCR contained two primers that bind to either side of the SNP and amplify a fragment that is always present (Arms-GenF: CGACGATGCCACAACTAGA and R12: AGTCTGCACTAATATCATGACCTC). Each PCR also contained a third allele-specific primer that allowed the amplification of a smaller fragment, provided the individual concerned had a copy of the corresponding allele. These primers are Arms-Gly-RT (TAGTTTCACATAGCCTTTTCC) and Arms-Ser-RC (TAGTTTCACATAGCCTTTCCT). According to the presence or absence of the smaller fragment in the two different PCRs, we could deduce whether the individuals were homozygotes for the $SnFor^{Gly}$ allele, homozygotes for the $SnFor^{Ser}$ allele, or heterozygotes. In 2 years of collection (2012 and 2013), 143 individuals were genotyped.

Pharmacological treatment

Activation of PKG

The larvae were treated with the PKG activator 8-Br-cGMP (Fluka Chemie AG, Switzerland), by ingestion. This molecule crosses cellular membranes and is relatively resistant to deterioration by the enzymes responsible for the deterioration of the endogenous cGMP (phosphodiesterases) (Ben-Shahar et al., 2003). Second-instar larvae were collected from several egg masses to reduce the probability of siblings, and individuals of homogeneous size were chosen preferentially (caterpillars were individually weighed before and after the experiment). They were isolated in 2 ml Eppendorf tubes. Every tube contained rearing diet supplemented with 8-Br-cGMP at a given concentration, and was changed every day. A range of concentrations was chosen from the literature [honeybees (Ben-Shahar et al., 2002); ants (Lucas and Sokolowski, 2009)] to estimate a possible dose effect: 0 µmol l^{-1} (control, n=40), 250 µmol l^{-1} (n=24), 500 µmol l^{-1} (n=40) and 1000 µmol l^{-1} (n=24). The treatment was applied for 3 days, and caterpillars were weighed every 24±1 h in order to control for a possible effect on growth. At the end of the treatment, the locomotor activity linked to foraging behaviour of the insects (then third-instar) was individually tested in the 'actimeter' (description below) for 48 h.

Activation of PKA

The larvae were treated with the PKA activator 8-bromo-cyclic adenosine monophosphate (Sigma-Aldrich) by ingestion. We applied the same procedure of non-invasive oral chronic treatment as described above. The following concentrations were tested: 0 (control), 250, 500 and 1000 µmol l^{-1}; n=40 per group (Ben-Shahar et al., 2002). At the end of the treatment, the larvae were individually tested in the 'actimeter' for 48 h.

The 'actimeter' system for automated recording of larval foraging activity

This system was designed by Chardonnet et al. (Chardonnet et al., 2012) from a device initially developed for the fruit fly, the DAM system (Drosophila Activity Monitoring System, DAM2®; Trikinetics, Waltham, MA, USA) (Freeman et al., 2010; Hirsch et al., 2009; Huang et al., 2009). The actimeter apparatus is made of tubular units (200×7 mm i.d.), each provided with three infrared photoelectric detectors that automatically count and record larval movement between two food patches (Fig. 3). The tubular configuration reproduces a stem-like confined structure, and the artificial diet inserted at both tube ends creates two food patches. The food patches were made of rearing diet; one patch is enough to feed a caterpillar during the whole experiment (i.e. the food patch was not entirely consumed when an immobile larva stayed in a unique patch for the 3 days of the experiment). To compare locomotor activity between the third-instar larvae from the different treatment groups, a 'leaving the patch' event was recorded each time a larvae crossed at least two of the three consecutive infrared beams. The device allows us to test up to 32 larvae simultaneously. The order of the larvae from the different groups in the actimeter was systematically alternated to avoid a position effect. The tests were performed in the same environmental conditions as for rearing (26°C, 70% relative humidity, and a 14 h:10 h light:dark cycle). The data were collected using DAMSystem collection® software (TriKinetics, Waltham, MA, USA).

Gene–behaviour correlation

In order to test for a correlation between *Snfor* genotypes and foraging phenotypes, we tested the foraging activity of 179 third-instar larvae from the studied polymorphic population of *S. nonagrioides*. The third-instar was chosen as it corresponds to the instar when the insects begin to leave the hatching plant and forage for new stems in their natural environment (Jepson, 1954). It corresponds to the middle of larval development, which includes six to seven larval instars until feeding cessation and pupation. The behavioural analyses were conducted for 72 h according to the 'actimeter' protocol described above. At the end of the behavioural test, the larvae were frozen in liquid nitrogen and then sequenced using the ARMS technique (they were then at the fourth-instar).

Comparison of *Snfor* gene expression between allelic variants

Two strains homozygous for *Snfor*, $SnFor^{Gly/Gly}$ and $SnFor^{Ser/Ser}$, were each initiated from 15 homozygote founder couples taken from the laboratory-reared polymorphic population and genotyped after reproduction. For the whole-body analysis, 30 fourth-instar larvae per strain were tested. For the brain analysis, 64 fourth-instar larvae per strain were tested. In this condition, the brains (deutocerebrum) were pooled in groups of eight (i.e. eight replicates for each population). The live larvae were retrieved from the artificial diet at the fourth-instar stadium, a stage usually reached during behavioural testing protocol, and immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction.

Total RNA was extracted from whole larvae using TRI Reagent (Sigma-Aldrich). The brains were dissected in the RA1 buffer and total RNA was extracted using the NucleoSpin RNA XS kit (Macherey Nagel). In both conditions, a DNase treatment (DNase I recombinant, Roche) was also performed. The integrity of each RNA sample was verified with the Experion Bio-Analyzer (Bio-Rad). All the samples showed a clear peak, without any sign of RNA degradation. One microgram of RNA extracted from the whole body was used to synthesize 20 µl cDNA for each sample using the iScript cDNA Synthesis Kit (Bio-Rad). In the brain extract analysis, 400 ng were used to synthesize cDNA. A 203 bp fragment of the *Snfor* gene was amplified with the primers QFORF (CATCGACCGGGAGACCTT) and QFORR (TCCTGCTTGAGTCGCTCTG). Three reference genes were used to normalize the samples: *rpL13* (170 bp; QRPL13F3-TACCGCAGAAAACAAAACAGGAT/QRPL13R3-GCGGGGTTAAGACCAGATGC), *rpS3* (121 bp; QRPS3F3-GCTGAATCGCTCAGATACAAACT/QRPS3R3-GCTTGCCAGAGACTACCACCT) and *rpL17* (171 bp; QRPL17F3-TTTGGCACCACACAAGGTC/QRPL17R3-GGCACGGTATGTGCGTCTG). The PCR efficiency was calculated for each gene using a calibration curve with four dilution points using a pool of cDNA samples. In the whole-body extract analysis, the efficiencies were 92% for *Snfor*, 96% for *rpL13*, 99% for *rpS3* and 95% for *rpL17*. In the brain extract analysis, the efficiencies were 107% for *Snfor*, 99% for *rpL13*, 96% for *rpS3* and 91% for *rpL17*.

PCR was carried out in 10 µl, with 1X FastStart Universal SYBR Green Master (Roche), 0.3 µmol l^{-1} of each primer and 2 µl of 10-fold-diluted cDNA for the whole-body extracts. The same mix was used for the PCR on brain extracts, but using a fivefold-diluted cDNA. All samples were

amplified in duplicate, in order to check the consistency; a negative control was also included for each gene. The gene-specific primers were designed to span an intron, in order to avoid genomic DNA contamination in the analysis. For *Snfor* and *rpL17*, genomic DNA could not be amplified with the primers and amplification protocol we used; for *rpS3* and *rpL13*, the fragment amplified with genomic DNA was approximately 900 bp and so any contamination would be revealed by the melting curve analysis. Therefore, non-reverse transcript controls were not included in our experiment.

The PCRs were run on a CFX96 (Bio-Rad) – the amplification protocol was 95°C for 10 min and then 45 cycles of 95°C for 10 s and 60°C for 30 s – followed by a melting curve. All melting curves showed a single peak, suggesting specificity and homogeneity of the amplifications. The quantification cycle (C_q) values were determined based on the single threshold mode. All negative controls had a C_q of 0. The mean C_q standard deviation observed between duplicates was 0.055.

The stability of the reference genes was checked with Bestkeeper software (Pfaffl et al., 2004). The differences between the *Snfor* transcript concentrations were determined using the $\Delta\Delta C_q$ method [ABI User Bulletin #2 (11-15)]. The statistical significance of the difference in *Snfor* expression observed between the two populations was tested using REST 2009 software (Pfaffl et al., 2002).

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Competing interests

The authors declare no competing financial interests.

Author contributions

F.C. wrote the paper, performed behavioural and pharmacological experiments, and part of the molecular analyses, and ran data analyses. C.C.-D. performed the phylogeny of PKGI, most molecular experiments and wrote the related method sections. B.C. worked on cDNA sequencing and analysis. N.J. worked on the link between individual genotype and behavioural phenotype. M.H. suggested the genotyping method and performed the protein alignments. B.L.R. identified the insects, documented the ecology and biogeography of *S. nonagrioides*, and edited the manuscript. J.-F.S. provided general laboratory facilities and support and edited the manuscript. L.K. conceived and supervised the study, and contributed to data analyses and writing of the paper. N.J., F.C., B.C. and L.K. collected field insects and contributed to rearing.

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