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The spread of resistance to imidacloprid is restricted by thermotolerance in natural populations of *Drosophila melanogaster*

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Abstract

Imidacloprid, the world's most utilised insecticide[1], has raised considerable controversy due to its harmful effects on non-pest species[2–6] and there is increasing evidence showing that insecticides have become the primary selective force in many insect species[7–14]. The genetic response to insecticides is heterogeneous across population and environment[15–17], leading to more complex patterns of genetic variation than previously thought. This motivated the investigation of imidacloprid resistance at different temperatures in natural populations of *Drosophila melanogaster* originating from four climate extremes replicated across two continents. Population and quantitative genomic analysis, supported by functional tests, demonstrated a polygenic basis to resistance and a major trade-off with thermotolerance. Reduced genetic differentiation at resistance-associated loci indicate enhanced gene flow at these loci. Resistance alleles showed stronger evidence of positive selection in temperate populations compared to tropical populations. Polygenic architecture and ecological factors should be considered when developing sustainable management strategies for both pest and beneficial insects.

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26 **Introduction**

Many organisms recurrently exposed to pesticides develop resistance, causing major issues for the
28 sustainable control of pests and pathogens. Resistance emerges through many, if not any, mechanism
that stop or limit the toxic compound, or toxic derivatives, from reaching its molecular target. However,
30 understanding the extent to which ecological and evolutionary context shapes the adaptive response to
insecticides remains challenging. While resistance emerging through mutations of major effect has
32 been extensively described[18–20], resistance that has a multifactorial basis has often been overlooked.
Recent large-scale genomic projects underscore findings that the amplification of gene families often
34 contributes to insecticide resistance[21–23] and that, alongside new mutations, standing polygenic variation
and ecological factors play an important role globally[24,25]. A quantitative basis to resistance is a
36 theoretical requirement to support the many cases of recessive resistance, and also helps explain why
resistance to new pesticides is occurring increasingly faster since the introduction of modern chemical
38 controls (penicillin, DDT and 2,4-D for microbes, insects and weeds, respectively)[26–29].

Under steady selection, alleles conferring resistance are expected to go to fixation and selective sweeps
40 have indeed been widely observed for loci underlying resistance, one of the best examples being the
Cyp6g1 locus conferring resistance to DDT and many other chemicals in *Drosophila melanogaster*[8,18].
42 This locus has experienced a sequence of selective sweeps which increased the frequency of resistant
alleles[30,31]. However, the ancestral susceptible allele was still evident in recent samples of natural
44 genetic diversity, showing that highly favoured resistance alleles might not always fixate[32]. The
persistence of such polymorphism suggests evolutionary trade-offs between resistance and other
46 ecological factors[16,33] which are poorly understood particularly for multifactorial resistance.

Resistance mutations often occur in genes that exert a primary, ancestral function (e.g the nervous
48 system for insect or the photosystems for plants) while resistance is a secondary, derived function.
Selective forces may act differently on resistance genes recruited from different pathways and induce a
50 fitness cost for bearing the resistance allele and trade-offs may vary among populations depending on

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local adaptive pressures[34–36]. However, evidence of fitness costs associated with resistance are not that
52 commonly reported in natural pest populations[37–40]. This could be due at least in part to a complex eco-
evolutionary context that is difficult to reproduce in the laboratory and experimental measurements of
54 fitness may not reflect those that are relevant in field conditions[41]. A way forward is to combine
experiments that apply specific types of environmental variation with the use of population
56 backgrounds that are specifically adapted to particular environmental conditions. The detection of
trade-offs can then be facilitated by harnessing whole-genome sequencing approaches and a well
58 characterised set of responses to experimental perturbations.

At the species range scale, populations evolve across multiple environments under different selection
60 pressures[16]. This is particularly the case for widespread species including pests and disease vectors[42,43].
D. melanogaster is a cosmopolitan and synanthropic species that has a broad range and bears abundant
62 diversity within natural populations[44], ideal for testing complex evolutionary questions. Wild
populations vary in their responses to temperature[45] and insecticide[30] with potential parallels across
64 continents[46] so it is now timely to investigate interactions between multiple genetic and environmental
factors with population genomic resolution.

66 Here we explore how multiple field populations of *D. melanogaster* which have undergone long-term
selection in different climates have dealt with the novel and ubiquitous short-term selection pressures
68 associated with pesticides. Using a parallel sampling across the climate extremes of two continents, we
build on the unmatched resources existing in *D. melanogaster* to comprehensively analyse the effect of
70 Single Nucleotide Polymorphisms (SNPs), Insertion and Deletions (InDels), Transposable Elements
(TEs), Copy-Number Variants (CNVs) and symbiotic micro-organisms on the genetic architectures of
72 temperature and insecticide resistance. This provides a test of whether genetic differentiation observed
for resistance genes interacts with thermotolerance at the phenotype and molecular level.

74

Results

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76 *Longevity in response to combined insecticide and temperature stress*

In regions of extreme temperature and precipitation (Fig. 1A-B), 16 natural populations of *Drosophila*
78 *melanogaster* were sampled outdoors in autumn with two collections per climate zone and per
continent (Table S1). Individual flies (n=12,770) from 400 isofemale lines (i.e. the progeny of a single,
80 mated field-collected female fly) were tested for longevity within 25 generations from collection, and
were either exposed or not exposed to 1000ppm of imidacloprid diluted in sucrose media, at either
82 20°C or 30°C. Imidacloprid exposure reduced longevity by 83.9 hours (SE=4.9) producing a non-acute
response while increasing the temperature from 20°C to 30°C reduced longevity by 118.1 hours
84 (SE=4.1). The flies originating from hot climates had longer lifespan in the heat (ANOVA, n=7801,
$F_{1,7799}$=10.17, P=0.00143, Fig1C) consistent with anticipated climate adaptation, but had shorter
86 lifespan when exposed to imidacloprid (ANOVA, n=10612, $F_{1,10610}$=11.13, P=0.000854, Fig1C). The
climate zone of origin as well as the continent of origin had a significant effect on longevity, however
88 the models including population and isofemale line effects explained a greater proportion of variance
(Table S2). 58% of the variance in time-to-death was explained by isofemale genotype effect and 32%
90 by population effect, suggesting that genetic variation for longevity segregates more within population
than among them. The responses to elevated temperature and imidacloprid computed from the effects
92 of linear model with the best fit to the data were strongly negatively correlated, both at the population
(ρ=-0.91, Fig. 1D) and at the isofemale genotype level (ρ=-0.68, Fig. 1E), revealing a trade-off between
94 insecticide and elevated temperature tolerance within and among populations. The Australian
populations were more resistant to imidacloprid than the American (Fig. 1D) with the exception of the
96 population from Victoria (Australia).

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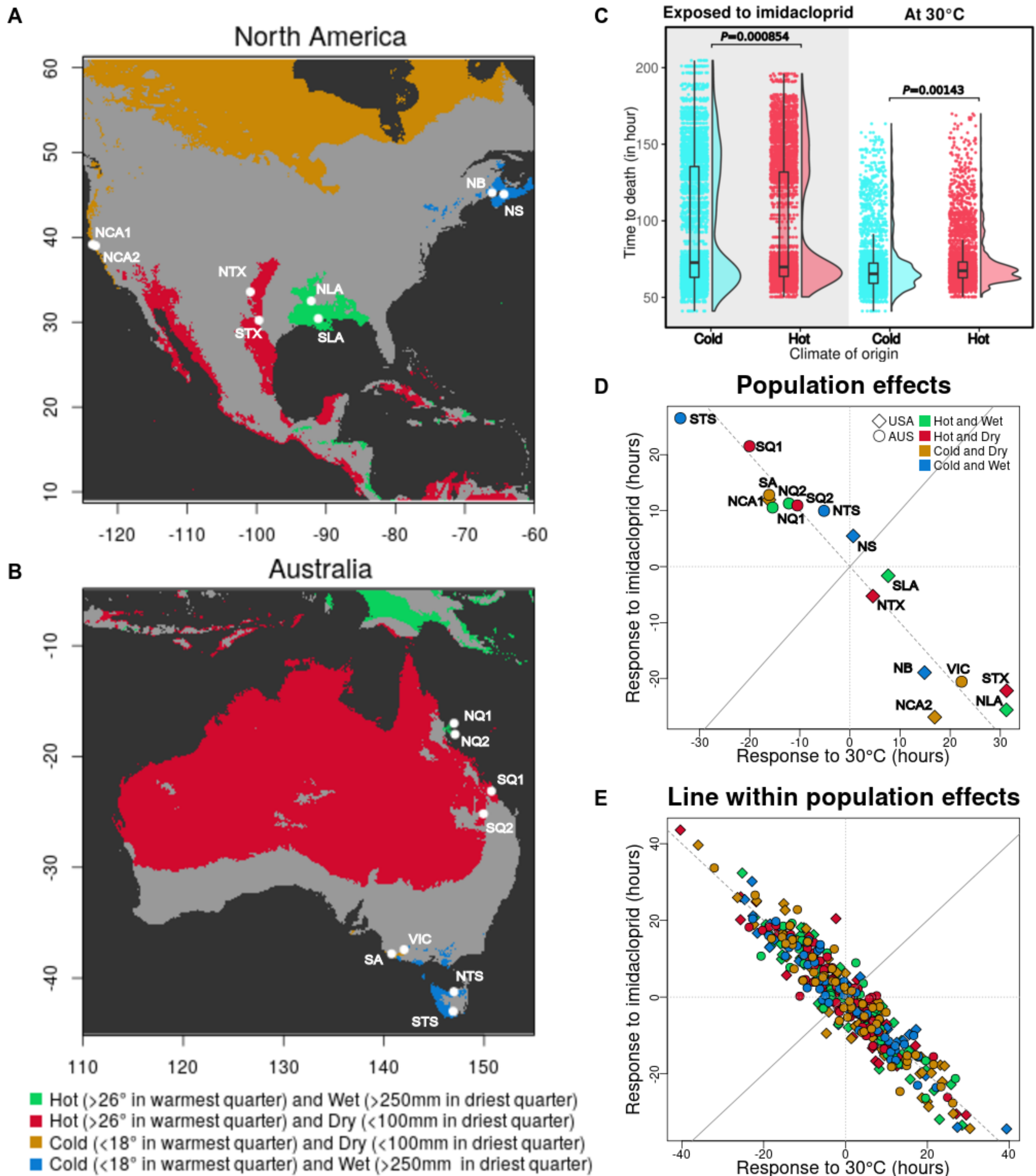

Figure 1: A-B) Origin of the populations sampled and location of extreme climates in North America and Australia. C) Difference in the longevity of individual flies exposed to 1000ppm imidacloprid or to 30°C depending on their climate of origin. D-E) Mixed-linear model effects of population or isofemale line within population variation on longevity under elevated temperature (30°C) **and insecticide stress** (1000ppm imidacloprid)**.** Population labels are: NB: New Brunswick, NCA1: North California 1, NCA2: North California 2, NLA: North Louisiana, NQ1: North Queensland 1, NQ2: North Queensland 2, NS: Nova Scotia, NTS: North Tasmania, NTX: North Texas, SA: South Australia, SLA: South Louisiana, SQ1: South Queensland 1, SQ2: South Queensland 2, STS: South Tasmania, STX: South Texas, VIC: Victoria.

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98 *Molecular variation associated with climate and resistance to imidacloprid*

For each population at each temperature, every fly exposed to imidacloprid was assigned into one of
100 five pools of resistance that covered the complete phenotypic distribution; each pool of genomic DNA
was sequenced. The sequence reads were mapped to the *Drosophila melanogaster* "hologenome",
102 which includes the reference genome, and those of six of its most common microbes[47] to call SNPs, TE
insertions and CNV. SNP-based polymorphism and genetic diversity were consistent across populations
104 (average π=0.0058 and $\theta_{Watterson}$=0.0059 for the autosomes, Table S2) with the exception of the North
California 1 population that showed about two-fold more diversity (π=0.0116 and $\theta_{Watterson}$=0.0134).
106 Genome-wide average estimates of π and $\theta_{Watterson}$ were not different (paired t-test, n=16, t=-1.746,
P=0.101), suggesting populations were sampled at a demographic equilibrium without recent intense
108 selective or founding events. Neither the temperature nor the precipitation at the location of origin was
correlated with the level of polymorphism (GLM, n=16, P>0.15).

110 Using SNP alleles tagging cosmopolitan chromosomal inversions[48], the *In(3R)Payne* inversion was
found to be more prevalent in Australia and showed clinal variation from high frequency in North
112 Queensland to low frequency in South Tasmania, consistent with previous findings[49] (Table S3). The
In(2L)t and *In(3R)Payne* inversions were more frequent in hot climates (three-way ANOVA testing
114 continent, temperature and precipitation regime of origin, n=16, *In(2l)t:* $F_{1,11}$=22.1, P=6.53x10^{-4} and
In(2l)Payne: $F_{1,11}$=22.0, P=6.63x10^{-4}) and *In(3R)Mo* in wet climates ($F_{1,11}$=4.973, P=0.0202). The only
116 common microbe differentially present across populations was *Acetobacter pomorum* which was more
frequent in North America (Table S4, $F_{1,11}$=6.730, P=0.0249) and in cold climates in general
118 ($F_{1,11}$=5.643, P=0.0368). Interestingly, the presence of pathogenic bacteria *Pseudomonas entomophila*
and *Gluconobacter morbifer* correlated with the increased lifespan of flies exposed to imidacloprid
120 from North Tasmania and in North California 1 and South Australia (GLM, 5 pools, P=0.008, P=0.001
and P=0.006, respectively), always at 30°C. We also detected a positive effect of *Wolbachia pipientis*

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122 on longevity at 30°C in North Louisiana and North Texas (GLM, 5 pools, P=0.005, P=0.007).

Genome-Wide Association (GWA) between the SNPs and TEs and imidacloprid resistance revealed the
124 influence of two major loci (Fig. 2A-B). These two loci were identified in 22 and 21 out of the 32 GWA
tests (16 populations tested for two temperatures, Fig. S2), and were located within the coding
126 sequence of the *Paramyosin* (*Prm*) gene and within the coding sequence plus 60kbp upstream of the
Nicotinic-Acetylcholine Receptor Alpha 3 (*nACHRα3*) gene, the latter encoding a subunit of the
128 nicotinic-acetylcholine receptor, imidacloprid's molecular target[50]. CRISPR deletion alleles of
nACHRα3 increased imidacloprid susceptibility while heterozygous MiMIC[51] disruption for *Prm*
130 increased susceptibility only at 20°C (Fig 3).

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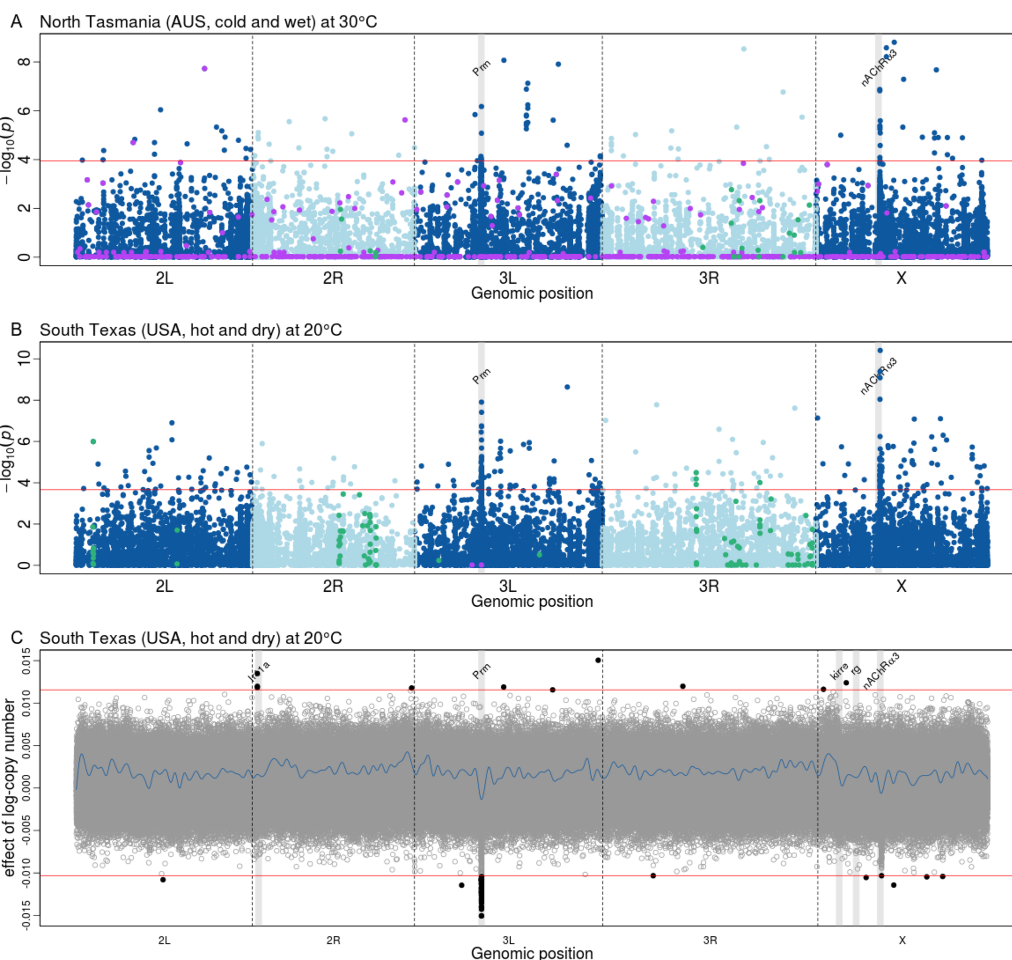

Figure 2: A-B) Manhattan plots indicating the genomic position of polymorphisms associated with imidacloprid resistance. Blue dots are SNP and small InDels, green dots are Inversion-tagging SNPs, purple dots are transposable elements and the red line indicates the FDR=5% threshold. Vertical shades of grey indicate the position of the two main candidate genes, *Paramyosin* and *N-Acetylcholine receptor α 3* located in position 3L:8,733,456-8,745,162 and X:8,277,227-8,438,674 of the *D. melanogaster* genome v6. **C) Effect of CNV on imidacloprid resistance by windows of 266bp.** Positive values indicate a greater number of copies increasing resistance. Grey and black dots are non-significant and significant CNV, respectively; the blue line represent a smoothed spline curve joining
160 the individual dots (smoothing parameter λ=0.03)**;** the red lines indicate the FDR=5% threshold. Vertical shades of grey indicate the position of the five main candidate genes: *Ionotropic receptor 41a*, *Paramyosin*, *Kin of Irre*,
162 *Rugose* and *N-Acetylcholine receptor alpha 3*.

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164 16 of the 26 TEs associated with imidacloprid resistance belonged to the R1A1-element family (analysis restricted to the Australian populations which have higher sequencing coverage overall). All
166 but one R1A1-element located in position 2R:19,900,676-19,901,175 (*D. melanogaster* v6 assembly) in the North Tasmania population were rare with a frequency lower than 0.06, suggesting that in our
168 data, TEs did not heavily contribute to the variation detected for insecticide resistance (Fig. S1). 62 inversion-tagging SNPs out of 473 were significantly associated with imidacloprid phenotypes in five
170 American and seven Australian populations (Fig. S2) with 41 out of these 62 SNPs decreasing resistance to imidacloprid. 34% of all SNP tagging *In(2L)t* and 72% of all SNPs tagging *In(3R)Payne*
172 showed a significant association in at least one GWA test which is higher than expected by chance. GWA tests between CNVs and imidacloprid resistance in the Texas populations further supported the
174 presence of major effects at the two major loci detected through SNP-based GWA with fewer copies corresponding to increased imidacloprid resistance which is the opposite of knock-out mutation effects
176 (Fig. 2C). However, the three CNVs most commonly influencing resistance across populations (in at least 10 out of 32 GWA tests, Fig. S3) all corresponded to higher copy-number increasing longevity.
178 These CNVs located in position 2R:4,917,519-5,021,955, X:2,740,384-3,134,532 and X:5,085,759-5,254,864 colocalised with the *Ir41a*, the *kirre* and the *rg* genes, respectively.
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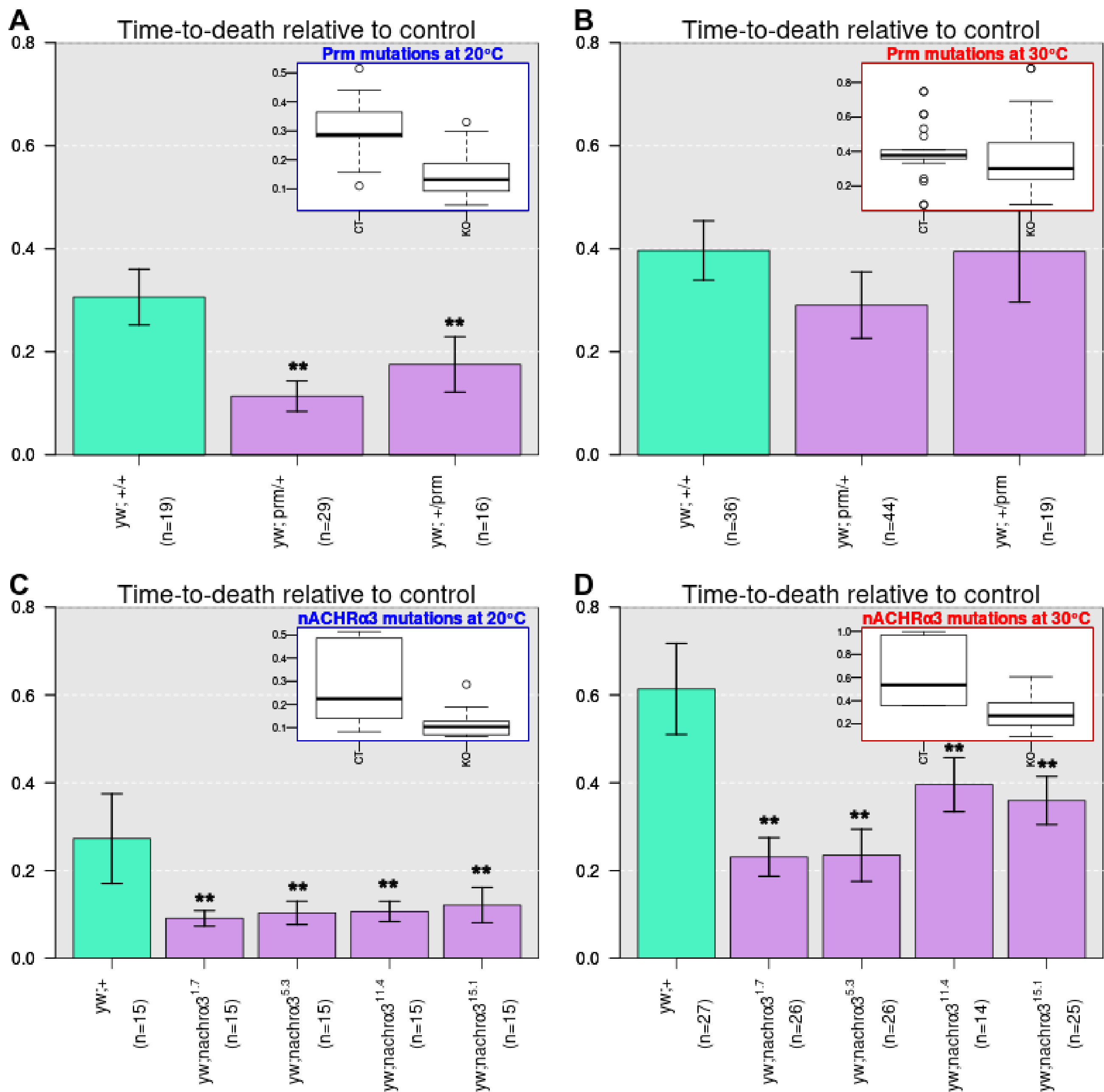

Figure 3: A-D) Mean longevity +/- standard error of flies exposed to imidacloprid relative to control (y-axis) **for wild type and mutants in candidate genes.** The top-right box in each panel is a scatter-boxplot recapitulating the difference in genetic effect between mutant and wildtype for each gene and temperature comparison in the same unit of relative longevity with respect to control; the thick shows the mean effect, the box shows the interquartile distribution and the whiskers, the 5%- and 95%-tile distribution. Test for *Prm* are shown in panel A and B, *nAChRα3* in panel C and D tested at 20°C (panel A and C) or 30°C (panel B and D)**.** **: P-value<0.01 (t-test).

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192 *Patterns of population differentiation and selection among populations*

The genetic differentiation (F_{st}) between populations was correlated with geographic distance,
194 consistent with isolation by distance (r_{Mantel}=0.23, P<0.001) with no influence of latitude (distance from
the equator, r_{Mantel}=0.03, P=0.442). Overall less than half of the genetic differentiation was explained by
196 climate distance ($r_{partial\ Mantel}$=0.11, P=0.018). However, two regions at the top of chromosome 2L and
bottom of chromosome 3R collocating with the *In(2L)t*, *In(3R)Mo* and *In(3R)Payne* inversions showed
198 high genetic differentiation driven by climate consistent with previous findings[47,49,52](Fig. 4A-B).
Conversely, the genomic regions where candidate genes are located show little evidence of climate
200 differentiation. We found that the average F_{st} value for genomic intervals containing resistance
candidates identified across multiple GWA tests, was lower than expected (Monte Carlo simulation,
202 P<0.006, Fig. 4D), highlighting minimal genetic differentiation for resistance candidate genes and
supporting the hypothesis of an increased gene flow for these loci.
204 The evolutionary state of each allele, either ancestral or derived, was inferred by remapping the
genome of the four most closely related species of *Drosophila* for which complete genomic sequences
206 are available (*D. simulans*, *D. yakuba*, *D. erecta* and *D. sechellia*). For 23 out of 32 GWA tests, the
resistance alleles predominantly corresponded to derived allele states while susceptible alleles were
208 ancestral (χ^2, maximum P<0.026 for these 23 GWAS). Three out of four tropical populations did not
follow this pattern (both Louisiana and North Queensland 1 populations), with derived alleles
210 conferring insecticide susceptibility at all temperatures (showed in the green box in Fig. 4C). This was
also true for the South Texas, the North California 1 and the Victoria populations but only for the
212 temperature furthest from their expected climatic preference (20°C, 30°C and 30°C, respectively).
Using global freshwater pesticide data[53], we did not find an effect of the level of pesticide pollution at
214 the location of origin on insecticide resistance or the excess of derived allele conferring susceptibility.
However, the excess of derived alleles conferring susceptibility positively affected the trade-off by
216 increasing longevity at 30°C over longevity exposed to imidacloprid (GLM, P<0.0005).

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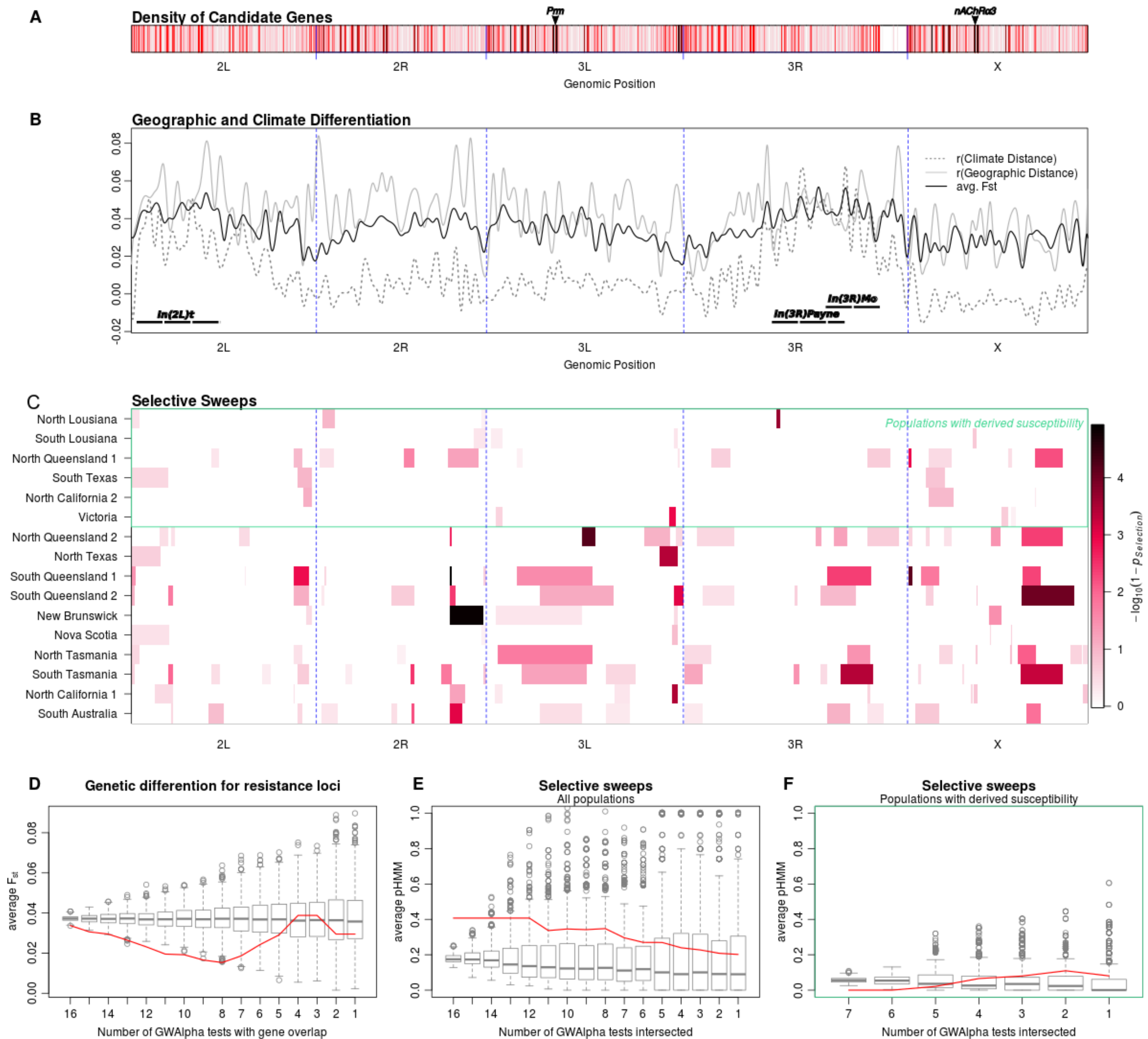

Figure 4: A) Density of candidate genes along the genome. Darker colours indicate higher density of candidate genes; The position of the two main canadidate gene *Prm* and *nAChRα3* is shown with an arrow **B) Average Fst and partial Mantel r statistic for correlations between Fst and geographic/climate distance over the genome** (sliding window of 5Kbp with smoothing parameter λ=0.05). The position of the three inversion associated with decayed resistance and increased climate adaptation *In(2L)t*, *In(3R)Mo* and *In(3R)Payne* is reported with long dashes. **C) Position of selective sweeps.** Selective sweeps were inferred based on the log-probability of transitioning from a neutral to a selected state (pHMM) inferred from a Hidden Markov Model based on site-frequency spectrum for each chromosome and each population. **D-F) Monte Carlo simulations testing the distribution of Fst among candidate genes** (solid red line) **compared to null genome-wide expectation** (box for interquartile and whiskers for inter-5%-ile distribution)**, the distribution of probabilities of selection in a Hidden Markov Model (pHMM) across all populations and the distribution of pHMM in populations with derived susceptibility**. The x-axis corresponds to the minimum number of GWA tests a given candidate gene was identified in (from the gene set found significantly associated in 16 or more GWA tests to the gene set found significantly associated in a one or more GWA test).

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Selective sweep analysis performed using Hidden Markov models of the allele frequency
234 spectrum[54] (Fig. 4C) strongly identified two regions in position 2R:20,779,995-20,989,995 and X:16,013,467-16,633,467 (top 1%-tile of selection probabilities averaged across populations) and a
236 region in position 3L:23,339,400-23,849,400 (top 5%-tile) but did not include *Prm* or *nAChRα3*. *Para*, encoding the molecular target of organochloride and pyrethroid insecticides[19], but never associated with
238 resistance in natural populations of *D. melanogaster*, was located in the regions of chromosome X showing evidence of positive selection (top 1%-tile of selection probabilities). Selection probabilities
240 among candidate genes identified in more than 13 out of 32 GWA tests were higher than expected (Monte Carlo simulation, $P<0.005$, Fig. 4E). The populations in which resistance alleles corresponded
242 to ancestral states showed overall less evidence of positive selection compared to other populations (t-test, $P=1.7x10^{-9}$). This pattern was accentuated for insecticide resistance loci: in the populations where
244 the derived alleles increased susceptibility, among which three are tropical (green box in Fig4C), there was a lower than expected probability of selection (Monte Carlo simulation, $P<0.001$, Fig. 4F).

246

Discussion

248 *Multifactorial bases to resistance*

Resistance to imidacloprid in natural populations of *D. melanogaster* was shown to be controlled by a
250 mixed genetic architecture involving major genes segregating across multiple populations and a polygenic background that exhibits a trade-off with thermotolerance. Of the two major genes we
252 consistently identified, *nACHRα3* is functionally well characterised. It encodes one of the seven *α* subunit of the N-acetylcholine receptor which is the known target of imidacloprid[55]. *Prm* has been less
254 extensively studied. In insects, it is known to encode important structural constituents of striated, obliquely striated and smooth muscles core thick filaments[56]. In *D. melanogaster*, *Prm* disruption was
256 shown to affect indirect flight muscle stiffness and power generation[57]. Two studies have shown up-regulation of *Prm* in the context of insecticide resistance; in *Tribolium castaneum* exposed to

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258 insecticidal growth regulator diflubenzuron[58] and in permethrin-resistant *Aedes aegyptii* after Zika virus infection[59], respectively. The involvement of *Prm* could be a neuro-muscular compensation of a post-
260 synaptic dysfunction as observed in human[60,61], and in this case, it could act after the binding of imidacloprid or perhaps ameliorate the *nACHRα3 mutation.* The two major candidate genes from our
262 study primarily point to target-site modes of resistance and this mechanism is underscored by significant enrichment of nervous system genes in the GWA analysis (Fig. S4, coupled with functional
264 evidence for *ank* and *Pde9*, Fig. S5). Conversely, we only identified two genes belonging to the canonical detoxification genes family, and neither of these (*Cyp311a1* and *Cyp4s3*) are strong
266 candidates for having roles in insecticide metabolism. The P450 *Cyp6g1*, that has previously been associated with imidacloprid metabolism[62–64], was not detected in our analysis and inspection of a SNP
268 tagging the CNV believed to be causal for the resistance (2R:12185332), is fixed in almost all Australian populations (the exception being a South Australian population) and was at high frequency
270 in the north American populations we sampled (0.71 to 0.97). The analysis of microbial "hologenomes" adds another likely contributor to the multifactorial basis to variation in insecticide mortality. The
272 microbes associated with resistance were all pathogenic, contrary to the climate- or continent-associated ones. This line of evidence leans toward a priming effect of immune defence
274 mechanisms[65] as opposed to the more common hypothesis of enhanced holometabolisation of toxic compounds being mediated through microorganisms[63,66–68].

276

Intra-genomic conflict could slow the evolution of resistance

278 We have probed multiple sources of molecular variation and showed that different classes of genomic change contribute to survivorship on insecticides and that they differ between temperatures. In
280 particular, copy-number variation can be a major mechanism for resistance evolution[23,30] and in this study, CNVs collocated with the two major resistance loci. While no significant pattern emerged
282 through the analysis of transposable elements, cosmopolitan genomic inversions showed more

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consistent effects. *Payne* and *Mo*, the inversions associated with increasing susceptibility in our study,
284 are likely to preserve gene complexes involved in climate adaptation[47,49]. These recombination-limiting
genomic structures may also slow the pace of evolution, particularly when a novel selection pressure
286 such as an insecticide interacts with a long-term one such as environmental stress. The fixation of a
resistance allele can thus be considerably slowed down by the genomic context and result in the
288 persistence of polymorphism irrespective of directional selection on the resistance allele. Nonetheless,
this study also suggests that the genetic response to imidacloprid is conditional to the environment to
290 which insects are pre-adapted. Insecticide resistance is thus fundamentally a complex trait likely to be
both affected by genotype-by-environment interactions and by selection acting on a specific locus with
292 different evolutionary potential. Due to a focus on variation segregating within populations, resistance
mutations that have already reached fixation or that are near fixation (such as *Cyp6g1* alleles in
294 Australian populations) do not arise in our genome wide association studies. However, a high fraction
of resistance loci showed intermediate frequency with low levels of population differentiation relative
296 to other variants at similar frequency across the genome. This suggests that gene flow is higher in
these regions and that could be explained by selection favouring the spread of such variants. This is
298 supported by the finding that imidacloprid resistance loci, notably those in temperate populations show
signals of selection in their allele frequency spectrum. Given recessivity is typical, the increase in
300 frequency of such polymorphisms foreshadow resistance outbreaks and motivates the identification of
similar polymorphisms in pest species.

302

The use of the eco-evolutionary context for managing resistance

304 Species that develop resistance to pesticides are typically widespread and they often are found to
exhibit local adaptations that initially appear distinct from adaptations to control chemicals[69]. However
306 here we have shown that the ecological genomic approach we have taken can identify important trade-
offs between contrasting selective pressures. The peri-domestic ecology of the model insect

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308 *Drosophila melanogaster* was used as an environmental sentinel and the wealth of molecular resources available to understand its biology provides a powerful platform for testing multiple hypotheses. While
310 it is well established that the same major insecticide resistance variants may arise independently in distantly related species (e.g. the same knockdown resistant amino acid mutations have occurred in
312 many insect species[24]), it is less likely that genes contributing to the polygenic component will be common between species. However, the polygenes of different species are more likely to share
314 molecular functions or act in common molecular pathways. Furthermore, the finding that the genomic architecture of insecticide resistance can point to evolutionary limits should encourage a systematic
316 targeting of genomic regions where differentiation is high and/or where recombination is limited in pest and vector insects (many pest insects do indeed harbour chromosomal inversions). Here we identified
318 such genomic regions by combining three experimental factors in the design of the study: rational sampling across climate extremes, traits being tested under multiple conditions and whole-genome
320 sequencing. The population sampling ensured the ecological context of pre-adaptation to distinct temperature regimes was retained to its best extent and the tests at different temperatures led to
322 physiological responses that increased the chance of identifying trade-offs. The pool-sequencing approach enabled efficient analysis of whole-genome data. The array of methods used in this study are
324 readily transferable to most species inhabiting diverse environments, regardless of whether they are pests, disease vectors or beneficial species affected by pesticides.

326

Conclusion

328 Knowledge of an insecticide-to-insect molecular interface alone, in the absence of a relevant environmental and genomic context, will likely not be enough to understand and predict resistance
330 outbreaks, and will not provide an appreciation of the interplay of environment variation, genetic constraints and evolutionary outcomes. Ecological barriers seem capable of limiting the spread of
332 functional genetic diversity, and such barriers need to be more systematically investigated across

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multiple populations and environments. Imidacloprid has been widely used in the last decades and has
334 received considerable attention for its effect on beneficial insects in recent years. The response to
imidacloprid in *D. melanogaster* is thus generally relevant even though the reductionist nature of the
336 experiments would have overlooked other factors such as resistance to multiple other insecticides and
climate variables which could add to the already complex situation described in our study. However,
338 the evolutionary response it promotes is likely to be very similar to that of other classes of pesticide and
the genomic mechanism of the response are also likely to be similar in other insects (inversion or
340 CNV). Understanding resistance evolution across tiers of biological organisation including populations
across the entire species range, genotypes within populations and explicit genomic context is thus
342 becoming the new challenge. With such information, there is an opportunity to develop an improved
understanding of the evolutionary trajectory of insecticide resistance in target pests and non-target
344 species.

346 **Methods**

Fly collections, maintenance and bioassay

348 Natural fly populations were collected outdoors in October 2013 in North America and in March/April
2014 in Australia, corresponding to early Autumn for each continent (Fig. 1A-b and Table S1). 25
350 isofemale lines were established from each population and reared in the lab under constant light and
temperature (25ºC) conditions on Bloomington cornmeal media. Flies were kept for less than 25
352 generations in the lab before performing the experiments.

For each population, five 4 to 7 day old individual female flies from each isofemale line were
354 individually exposed to an 1000ppm imidacloprid (Confidor®; Bayer Crop Science) diluted in 500mL
of a 2% agarose/5% sucrose + 50mg/L tegosept media. Controls were exposed to the same mixture
356 lacking imidacloprid. This design was tested at two different temperatures (20ºC and 30ºC) and
replicated three times with flies from different generations to avoid batch effects. For each replicate, the

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358 time-to-death of 125 individual flies (5 flies per isofemale line) was recorded every 12 to 18 hours. For each replicate of each population the dead flies were immediately frozen and distributed into five pools
360 (6.25%-, 24.5%-, 74.5%- and 94.75%-tile) based on their level of resistance as measured by time of death. This equates to 375 flies being distributed across 5 pools for each library synthesis (24, 72, 123,
362 72 and 24 flies respectively).

364 *Sample preparation and sequencing*

Total genomic DNA was extracted using phenol/chloroform/isoamyl alcohol. Total DNA was
366 quantified and 10ng was used to synthesise sequencing libraries using Illumina TrueSeq nano (Illumina, San Diego, USA) and barcode adapters kits (Agilent, Santa Clara, USA). Library quality and
368 DNA concentration was measured on an Agilent 2200 TapeStation using D1000 DNA tapes (Agilent, Santa Clara, USA). The 160 libraries (16 populations x 2 temperatures x 5 times-to-death)
370 corresponding to either the Australian or American samples were pooled and run on 7 lanes of the Illumina NextSeq 500 sequencer (Illumina, San Diego, USA) in 75bp paired-end reads mode.

372

Genomic analysis

374 Paired-end sequences in FASTQ format were trimmed to remove adapter and barcodes and aligned to the *Drosophila melanogaster* “hologenome” consisting of the reference sequence v5.40 plus common
376 microbes[47] using the Burrows-Wheeler Aligner (BWA v0.7.12-r1039) with the *mem* option and default parameters[70]. PCR and optical duplicates were removed using Picard v1.112 and reads locally realigned
378 around insertions/deletions using GATK v8.25. Alignment files in BAM format were merged for the different replicates using SAMtools and the resulting BAMs for the different pools of a given
380 populations and temperature test were synchronised into MPILEUP using SAMtools and SYNC files using Popoolation2 v1.201. SNP and InDels were called using a custom script ensuring a Phred base
382 quality score greater than q30, a minor allele frequency greater than 0.03, a base coverage greater than

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the 25%-tile of the corresponding pool. Finally, only SNPs present in both temperature tests (treated as
384 biological replicates) were called. The criteria of presence in both temperature tests ensured a robust
identification of polymorphisms.
386 TE were called using PopoolationTE2[71] with the *D. melanogaster* v5.40 genome masked for the TEs
present in the reference sequence and using a publicly available TE hierarchy for *D. melanogaster*
388 (https://sourceforge.net/projects/popoolation-te2/files/walkthrough-refgenome.zip/download). As for
the SNPs and InDels, the presence of a TE in a population was validated if detected for the two
390 temperature tests (biological replicates) within a window of 5kbp. Chromosomal inversion diagnostic
SNPs were retrieved from the literature[48], the corresponding positions were retrieved directly from the
392 MPILEUP file without further cutoffs and the relevant alleles were tested for association, but excluded
from the other polymorphism and population analysis. CNV were called based on read-depth using the
394 CNVkit software v0.9.3[72]. Frequency of symbionts in each pool was detected from the merged BAM
alignment file by comparing the average read depth over a given symbiont genome divided the average
396 read depth of the *D. melanogaster* genome using SAMtools and custom scripts.

398 *Statistical analysis*

Mixed-linear models were computed using the lme4 package of the R software. Nested models were
400 compared using an ANOVA procedure and compared using deviance and Akaike information criteria
(Table S2). The best model was:
402 Time-to-death = μ + β_1*Temperature + β_2*Insecticide + β_3*Temperature*Insecticide +
z_1*(Temperature+Insecticide | $_{\text{population}}$) + z_2*(Temperature+Insecticide | $_{\text{line}}$) + ε,
404 where μ is the intercept, β's are the fixed effects of increasing the temperature from 20 to 30ºC, the
fixed effects of increasing the imidacloprid exposure from 0 to 1000ppm in the and the effect of their
406 interaction, z's are the random effects of increasing the temperature or the insecticide exposure among
populations and among lines within population and ε the residual term.

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408 Pool-based genome-wide association tests were performed with the GWAlpha package[73] using maximum likelihood estimation and significance was calculated using empirical p-values with a
410 Benjamini and Hochenberg correction for multiple testing for a false discovery rate of FDR=5% on the loci whose coverage was within the interquartile range for a given pool. Candidate genes were
412 identified based on the excessive 95% proportion of associated SNPs or InDels in the neighbouring 5kbp window using the D. melanogaster genome annotation v5.57. Network and enrichment analysis
414 were performed using the R spider pipeline[74] under the model D2 (one missing connection allowed) using 2047 gene name entries for D. melanogaster from the KEGG and Reactome databases (1st of
416 December 2017). 225 gene identifiers out of 745 were recognised and 178 had a pathway information entry in the databases.

418 The Monte Carlo simulations used to test population genetic hypothesis were conducted by randomly shifting the circularised genomic position a 1000 times. The targeted summary statistic was then
420 computed for the 1000 permutations to draw the expected null distribution and compare it to the observed statistic to derive the reported p-value.

422

Population genetics analysis

424 For all population genetics analysis, each pool alignment file was down-sampled to even coverage for the 10 pool BAM alignment files for a given population (5 resistance pools x 2 temperatures) and
426 merged into a single BAM file. Estimates for π and θ where obtained from the down-sampled merged BAM files using the Npstats package[75] for windows of 5kbp only retaining polymorphism located in
428 positions with quality phred > q30. Pairwise F_{st} between each population were estimated using the same setup as for π and θ using the Popoolation2 package[76]. Mantel and Partial Mantel tests were performed
430 using the R\vegan package using 1000 permutations for p-value calculation. Climate distance of 0 corresponded to populations sharing both similar temperature and precipitation at their location of
432 origin, 0.5 when sharing only temperature or precipitation or 1 otherwise. Ancestral and derived states

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for each SNP and InDel polymorphism were determined by "shredding" the reference genomes for four
434 closely related *Drosophila* species (*D. simulans*, *D. yakuba*, *D. erecta* and *D. sechellia*) using the
Wgsim script (https://github.com/lh3/wgsim) generating 2.5M 500bp-long fragments for each species
436 that were re-mapped onto the *D. melanogaster* v5.40 genome using BWA. Polymorphic sites were then
called using the *call* function from the BCFtools package v1.3.1 (https://samtools.github.io/bcftools).
438 For each SNP/InDel position, the ancestral state was assigned if three out of five (including *D.*
melanogaster reference genome) states were identical or left undetermined otherwise. Selective sweeps
440 were detected based on probability of selection in a Hidden Markov Model[54] implemented in the Pool-
hmm package v1.4.3[77]. Allele frequency spectrum was computed for each chromosome using a random
442 10% of the sites that had a coverage greater than 10X from the down-sampled merged BAM file for
each population. We performed a sensitivity analysis to determine the transition probability *k* from
444 neutral to selected state and determined that for $k>10e^{-4}$ the set selective sweeps detected remained
consistent, so k was set to $10e^{-4}$.
446 All custom codes are deposited on GitHub (https://github.com/aflevel/IMIresist_vs_TEMPtol) and the
derived data are deposited on figshare (DOI:10.26188/5b592f305226b). Raw sequencing data
448 generated in this study are available upon request.

450 *Functional tests*

Fly stocks were ordered at the Bloomington Drosophila Stock Center and the crossing design used to
452 obtain the tested lines are reported in Table S7. Deletions of *nAChRα3* were generated using two small
guide (sg)RNAs flanking the targeted region designed using CRISPR Optimal Target Finder
454 (http://tools.flycrispr.molbio.wisc.edu/targetFinder/), ActinCas9 gDNA was checked to ensure no SNPs
existed and then the nAChRα3 Del sgRNA 1 (GTCGCCGCAAACGCACACGA) and nAChRα3 Del
456 sgRNA2 (GCCCATCGATATAAAGCTAT) were ordered as oligonucleotides and each ligated
separately into pU6-BbsI-gRNA plasmids as per the published protocol (http://flycrispr.molbio.wisc.

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458 edu/protocols/gRNA). These were co-injected (each plasmid at 200ng/µl in water) into ActinCas9 flies following a standard microinjection protocol. Given the *nAChRα3* gene is located on chromosome X,
460 injected adult male survivors were initially crossed back to AC9 and F1 males collected. A wing was clipped from males, DNA extracted and diagnostic PCR (Primers F
462 5'GTGTGACTGTATTGGTGCTG3' and R 5'CACACACAGTCTGATGGAGC3') for deletion events performed. Flies with products smaller than the expected size (3590kb) were individually crossed to
464 virgin female FM7 balancer flies and these were backcrossed to remove the FM7 balancer, producing the four independent homozygous deletion strains of *nAChRα3* used in this study (1.7, 5.3, 11.4 and
466 15.1). Off-target analysis is reported in section 4.1 of the Supplementary Data.

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658 RTG and SW performed the experiments; AFL analysed the data with contribution from AAH, RVR and Paul Battlay; AAH, Paul Battlay and TP revised the manuscript; AAH, MS, Phil Batterham, TP and
660 WC provided new genetic material.

662 **Competing interests**

The authors declare no conflicting or competing interests.

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