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Population genetics of *Lymnaea stagnalis* experimentally exposed to cocktails of pesticides

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Abstract Freshwater invertebrates may be regularly exposed to pesticides in agricultural landscapes, as water bodies such as ditches and ponds are the final recipient of many chemicals, through various transfer pathways. Local evolutionary impacts may hence be expected on populations, especially for species with a completely aquatic life cycle. We tested the hypothesis that exposure to combinations of pesticides used in crop protection programmes could increase the effect of random genetic drift in a non-target species, via demographic impacts. To do so, experimental populations of the freshwater snail *Lymnaea stagnalis* were created from a common genetic pool and exposed for three successive generations to treatments corresponding to two different crop protection plans (conventional and low pesticide input). Population genetic parameters were estimated in each generation on the basis of ten polymorphic microsatellite loci. Effects consistent with increased random genetic drift were observed for one sampling campaign performed in the third generation, i.e., decreased genetic variability and increased population differentiation in the group of populations exposed to the treatment programme whose demographic impact was the most effective on *L. stagnalis*. Otherwise, no clear pattern emerged and even opposed effects could be observed. All populations were found significantly inbred, mostly due to biparental inbreeding. Conversely, selfing was generally not significant, and did not express preferentially under high pesticide pressure. We conclude from this study that population genetics should be used very cautiously in the context of ecological risk assessment, especially when applied to natural populations.

Keywords Chronic exposure · Pesticides · Random genetic drift · Population genetics · *Lymnaea stagnalis*

Introduction

In the field, pesticides rarely enter in exclusive contact with their proper target organisms. Non-intentional exposure to such substances is thus common in agricultural landscapes, and aquatic organisms occupying habitats close to treated fields are at high risk of exposure to pesticides, especially when their whole life cycle occurs into the water. Water bodies such as ditches and small ponds are very common in agricultural areas and they contribute significantly to regional biodiversity (Williams et al. 2004; Davies et al. 2008). However, they are the final recipient for many chemicals from agriculture, including nutrients and pesticides, through various transfer pathways (drift, drainage, run-off; Liess 2002; Brown and Van Beinum 2009). Local evolutionary impacts on non-target organisms are thus expected, including processes related to selection and random genetic drift. Exposure to cocktails of pesticides, such as those used for crop protection programmes (e.g., combination of fungicides, insecticides and herbicides), even when repeated seasonally, may not trigger selective effects since it should be difficult for a population to adapt simultaneously to several selective forces. Indeed, although cross- and multiple resistance may occur towards molecules with different modes of action (e.g., Clark and Di

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Giulio 2011; Vogwill et al. 2012), a more general expectation is that all pesticides, if acting as selective agents, will not do it in the same way, especially on non-target species, and genetic trade-offs may be more likely to occur among traits that respond to different directional selective pressures. This basic principle is applied in strategies which combine different molecules with the objective of delaying the evolution of resistance in pests (see REX Consortium 2013). In the particular case of cocktails of pesticides and non-target species, the situation may come closer to the ecological case of fluctuating environment with alternating stressful and non-stressful periods, and the need for organisms to cope with ever changing conditions. This would be more likely to lead to stochastic phenotypic switching (see Kussell and Leibler 2005; Acar et al. 2008). Moreover, multiple exposure to potentially toxic substances with various modes of action may inflate random genetic drift in natural populations (Bickham et al. 2000; Medina et al. 2007; Bickham 2011; Ribeiro et al. 2012), simply through a demographic impact (population size, isolation), which in turn will slow down potential adaptive processes. This stochastic process, although non-specific to toxicants, should not be neglected in ecotoxicology, since it is known to reduce the ability of populations to respond adaptively to other pressures (e.g., Weber 1990; Lynch and Walsh 1998; Willi and Hoffmann 2009), and also because purifying selection (elimination of slightly deleterious mutations) is less efficient in small populations (Ohta 1992; Ellegren 2009), which can increase the risk of population extinction (extinction vortices; Gilpin and Soulé 1986).

Theoretical effects of random genetic drift have been widely explored by population genetic models (Hartl and Clark 2007). Basically, variation of allele frequencies in finite populations is due to the random sampling of alleles within the gamete pool at the origin of each next generation. Random drift causes irreversible fixation and loss of alleles in the absence of mutation or gene flow (as sources of new variants), and is inversely proportional to population size. Genetic variability is ultimately lost within populations, and because the process is random, population genetic neutral differentiation is increased (i.e., two different populations are not expected to fix the same allele at a given locus). Random genetic drift may also become predominant in very small populations and overwhelm the effect of selection. This should occur whenever the selection intensity $|s|$ is lower than $1/Ne$, with Ne, the effective population size (Kimura 1983; Keller and Waller 2002). Consequently, populations would suffer a fitness loss and, as abovementioned, even go extinct through genetic load accumulation (random drift load; Whitlock 2000). This should particularly affect alleles with small deleterious effects, as they will be more prone to behave as if neutral, compared to alleles with larger effects (e.g., lethals). Moreover, if recombination is absent (asexual species) or ineffective due to high inbreeding (e.g., highly selfing species), mutational meltdown and population extinction are anticipated (see Muller's ratchet; Lynch and Gabriel 1990). Finally, due to lower genetic variability and lower fitness (to which inbreeding can also contribute), small populations are expected to be less able to evolve adaptively to further environmental change (Willi et al. 2006).

Increased random genetic drift may thus clearly be considered as a negative impact. However, the specific forces responsible for observed patterns in nature are usually difficult to disentangle, especially because each population has its own characteristics and history. Therefore, the use of population genetic indices such as Wright's *F*-statistics, which denote genetic correlations within and among individuals and populations, may be hazardous in a context of decision making, such as ecological risk assessment. The present study aimed at testing the value of such neutral parameters as indicators of chemical effects on a freshwater invertebrate, using experimental populations set up from the same initial genetic pool and amount of variation. These populations were exposed for three generations to repeated pesticide treatments with multiple compounds, under two different treatment programmes (named hereafter conventional and low input) and compared to control populations. Both treatments were shown to have negative demographic impacts on *L. stagnalis* populations, and conventional treatment was the most effective (Auber et al. 2011; Auber 2011). Various population genetic parameters were compared across treatments over three generations. We expected populations showing higher demographic impact to have lower genetic variability, lower effective population size, and to be more differentiated, in consistency with the order control > low input > conventional. As *L. stagnalis* is a self-fertile hermaphrodite which preferentially outcrosses, we also compared population selfing rates across treatments, albeit expectations were more difficult to assess for this parameter. Although inbreeding depression (i.e., the fitness reduction of inbred progeny compared to the outcrossed one) has been shown to be more severe under stress (Armbruster and Reed 2005; Fox and Reed 2011), self-fertilization may be still favored under such conditions, if physiological adjustment to stress requires energy. Indeed, compared to selfing, outcrossing is most costly in terms of energy, due to the need to find a mate and to copulate, and this energy might be reallocated to stress response. Due to the fact that inbreeding depression is very weak in *L. stagnalis* (Puurtinen et al. 2007; Coutellec and Lagadic 2006; Coutellec and Caquet 2011), i.e., fitness performances of selfed and outcrossed progeny are very similar, this phenomenon may be quite relevant for this species under stressful conditions. Furthermore, such conditions

may become determinant for selfing evolution, since mate availability is impaired or outcrossing is too costly (see reproductive assurance hypothesis; Barrett 2002). Selfing and biparental inbreeding (outcrossing among relatives) may thus have specific evolutionary trajectories, and should be estimated separately (see Coutellec-Vreto et al. 1997). In the present study, as a direct environmental effect of stress, we might expect populations to express higher selfing rates under stressful than under benign conditions.

Materials and methods

Study species

Lymnaea stagnalis (Mollusca, Gastropoda, Heterobranchia, Euthyneura, Panpulmonata, Hygrophila) is a holarctic freshwater snail inhabiting ditches, ponds and lakes. It is a simultaneous hermaphroditic species which preferentially outcrosses.

Origin of experimental populations

A total of 16 experimental populations were created on a common basis, i.e., 40 adult snails originated from the same set of 40 families (one snail per family). These snails had been previously reared under laboratory controlled conditions as described earlier (Coutellec et al. 2011). Animals were individually marked with a honeybee plastic mark stuck to the shell, and introduced into a set of 16 outdoor mesocosms (see Auber et al. 2011; Coutellec et al. 2011). Briefly, aquatic systems were set up in cylinder-shaped 9 m^3 outdoor tanks in April 2008, and allowed to mature for 7 months before first pesticide treatments. During this period, mesocosms were naturally colonized by insects and other organisms, and inocula of phytoplankton, zooplankton, and benthic invertebrates (gammarids, asellids) were repeatedly introduced during the summer period. Introduction of laboratory-bred pond snails (*L. stagnalis*) was done in July 2008. For the sake of clarity, these will be called G_1 as in Coutellec et al. (2011).

Pesticide treatments

Treatments have been fully described earlier (Auber et al. 2011). In short, four treatment levels were set out, based upon the combination of two agropedoclimatic situations (which mostly differ in frequency and intensity of drainage events), and two types of crop protection programmes, "conventional", based on current practices in North Western France, and "low pesticide input", which was designed to reduce environmental risk, through the use of alternative products with an improved environmental profile. Both programmes include several treatments from November to May, which were applied from Autumn 2008 to the end of Spring 2010. In the present study, *L. stagnalis* populations initiated from the 40 adult founders were thus exposed to various cocktails of pesticides throughout the whole study period. Each treatment level was replicated in three mesocosms, and four mesocosms were used as controls (16 systems in total). The temporal analysis of macroinvertebrate community structure revealed that programme type was the most effective factor (Auber et al. 2011). Therefore, in the present study, agropedoclimatic scenarios were ignored and merged into chemical treatment levels, in order to increase statistical power.

Population genetics sampling

In Spring 2009, marked snails introduced in 2008 were retrieved and brought to the laboratory for other studies (life history traits; Coutellec et al. 2011). Not all mesocosms hosted well established populations of *L. stagnalis* all along the period. For example, one population was already extinct before any chemical treatment was applied, and never recovered. Another population (control 2) was almost extinct in 2008–2009, whereas it recovered in 2010. Therefore, sampling success varied widely across next generations (called hereafter G_2 and G_3). The number of individuals per sampling campaign and corresponding number of populations per chemical treatment are summarized in Table 1. It is to be noted that we assumed that no migration occurred between mesocosms throughout the experiment, due to the impossibility for snails to move other than passively from one system to another, and because the experimental staff regularly scrutinized the whole platform.

Microsatellite amplification and genotyping

Genomic DNA was chelex extracted from hemolymph or from a piece of foot tissue, with a proteinase K solution (10 mg/ml), and was heated during 2 h at 55 °C, then 10 min at 100 °C.

In the present study, 10 microsatellite markers were chosen, as they were previously described to be polymorphic in the original population (M.A. Coutellec unpublished results; Besnard et al. 2013): A2, B117, A112 (Knott et al. 2003; note that Table 1 in this paper displays reverse primers in the wrong sense), 2k27 and 2k11 (see Kopp et al. 2012), and EMLS05, EMLS10, EMLS26, EMLS41

Table 1 Summary information on samples collected in experimental populations of *L. stagnalis*

Sampling period	Generation	Nb of populations per treatment			Total Nb of individuals
		Control	Low input	Conventional	
Spring 2009	G_1	3	3	3	144
Summer 2009	G_2	3	2	3	292
Spring 2010	G_{3S}	4	5	2	498
Autumn 2010	G_{3A}	3	6	4	311

Table 2 Microsatellite loci used to describe the genetic structure of *L. stagnalis* experimental populations

Locus name	Dye	Genbank accession	Reference
A2[a]	6FAM	AY225955	Knott et al. 2003
B117[a]	VIC	AY225961	Knott et al. 2003
A112[b]	6FAM	AY225958	Knott et al. 2003
2k27[b]	VIC	EF208747	Kopp et al. 2012
2k11[b]	NED	EF208248	Kopp et al. 2012
EMLS05[c]	6FAM	JX287525	Besnard et al. 2013
EMLS10[c]	NED	JX287526	Besnard et al. 2013
EMLS26[d]	VIC	JX287529	Besnard et al. 2013
EMLS41[d]	NED	JX287531	Besnard et al. 2013
EMLS45[d]	6FAM	JX287532	Besnard et al. 2013

Primer sequences can be found in cited references

[a,b,c,d] Post-PCR multiplexes

and EMLS45 (Besnard et al. 2013). Labeling and multiplex conditions are given together with loci accession numbers in Table 2.

Fragment amplification was performed for each locus separately, using a nested PCR with the following set of primers: (1) sequence-specific forward primer with M13 at the 5′ end, (2) sequence-specific reverse primer, (3) universal fluorescent-labelled M13 primer (see Schuelke 2000). PCR were performed in 6 µL of reaction volume, containing 0.06 U Taq DNA polymerase and PCR buffer (GoTaq, Promega), 208 µM each dNTP's, 2.17 mM MgCl2, 0.055 µM, 0.055 µM sequence-specific forward primer with M13 at the 5′ end, 0.55 µM sequence-specific reverse primer and 0.57 µM universal fluorescent-labelled M13 primer (see dye used with each locus, Table 2). Touch-down PCR procedure was applied as follows: initial denaturation at 95 °C for 4 min, 20 cycles consisting of a denaturing step at 94 °C for 30 s, a touch-down annealing step from 65 °C to 55 °C for 30 s, and an extension step at 72 °C for 1 min, then 10 cycles consisting of a denaturing step at 94 °C for 30 s, an annealing step at 55 °C for 30 s, and an extension step at 72 °C for 1 min. A final extension step at 72 °C was performed for 5 min. Amplified fragments were then multiplexed (Table 2), and analysed using a capillary sequencer (ABI 3130xl).

Microsatellite scoring and diversity analysis

Allele peaks were scored using GENEMAPPER (Applied Biosystems). Allelic richness, expected and observed heterozygosities and F_{IS} values were estimated per locus and sample using the softwares GENEPOP (Rousset 2008) and GENETIX (Belkhir et al. 1996). Departures from HWE (heterozygote excess or deficiency) were tested using GENEPOP. Population selfing rate was estimated using RMES, a method based on the distribution of multilocus heterozygosity (David et al. 2007). As notoriously difficult to estimate, the effective population size, *Ne*, was inferred using two distinct single-cohort methods, one of which is based on linkage disequilibrium (program L_DN_E; Waples and Do 2008), and the other on sibship assignment (SA), as implemented in COLONY (Wang 2009). The former assumes random mating, whereas the latter accounts for inbreeding when specified, and should be less biased if the population is inbred. Population genetic differentiation (F_{ST}) was tested and population genetic parameters were compared among chemical treatment groups using F_{ST} (Goudet, 1995).

Results

As mentioned earlier, due to the lack of difference between agropedoclimatic scenario in terms of macroinvertebrate community dynamics, and also because *L. stagnalis* populations did not settle in every replicate mesocosm, only chemical treatment was tested in the present study. This allowed increasing statistical power with regard to this factor. Diversity and correlation parameters are given per population (across loci) in Table 3. Overall, regardless of chemical treatment, the mean allelic richness ranged from 1.9 to 2.5 alleles, and expected heterozygosity from 0.292 to 0.419. Observed heterozygosity varied from 0.194 to 0.357, and was in most cases lower than expected heterozygosity, leading to a significant positive F_{IS} value in at least one generation of each population. Population structure was tested per sampling campaign (Table 4). Global inbreeding (F_{IT}) was significant in all generations, while F_{IS} tended to decrease in generation G_3, ending up as non-

Table 3 Genetic diversity and correlation parameters estimated per *L. stagnalis* sample

Treatment	Population	Sample	*n*	*Ar*	*Hs*	*Ho*	F_{IS}	*P*
Control	1	G_1	18	2.3	0.349	0.276	**0.206**	<0.001
		G_2	37	2.2	0.358	0.250	**0.306**	<0.001
		G_{3S}	38	2.3	0.346	0.334	**0.030**	0.007
		G_{3A}	29	2.2	0.330	0.194	**0.403**	<0.001
	2	G_{3S}	55	2.3	0.378	0.332	**0.122**	0.006
		G_{3A}	21	1.9	0.292	0.246	**0.167**	0.003
	3	G_1	6	1.9	0.365	0.263	**0.296**	0.027
		G_2	40	2.3	0.357	0.246	**0.312**	<0.001
		G_{3S}	30	2.3	0.354	0.285	**0.194**	0.004
	4	G_1	18	2.3	0.335	0.271	**0.180**	0.019
		G_2	35	2.2	0.356	0.246	**0.314**	<0.001
		G_{3S}	14	2.1	0.385	0.252	**0.343**	<0.001
		G_{3A}	17	2.1	0.345	0.329	0.052	0.092
Low input	1	G_{3A}	20	2	0.328	0.316	**0.044**	0.018
	2	G_{3S}	54	2.3	0.375	0.346	**0.080**	0.016
		G_{3A}	16	2.1	0.331	0.252	**0.244**	<0.001
	3	G_1	19	2.1	0.281	0.246	**0.118**	0.001
		G_{3S}	46	2.3	0.330	0.329	−0.009	0.172
		G_{3A}	22	2.1	0.358	0.305	**0.143**	0.012
	4	G_1	11	2.1	0.368	0.354	0.039	0.121
		G_2	36	2.2	0.364	0.306	**0.163**	<0.001
		G_{3S}	58	2.3	0.388	0.302	**0.193**	<0.001
		G_{3A}	22	2.3	0.419	0.283	**0.327**	<0.001
	5	G_{3S}	54	2.5	0.368	0.276	**0.236**	<0.001
		G_{3A}	21	2.1	0.335	0.274	**0.186**	0.002
	6	G_1	15	2.3	0.380	0.263	**0.305**	0.001
		G_2	38	2.2	0.371	0.294	**0.211**	<0.001
		G_{3S}	42	2.2	0.359	0.259	**0.284**	<0.001
		G_{3A}	21	2.2	0.362	0.317	0.129	0.051
Conventional	1	G_{3S}	45	2.2	0.300	0.249	**0.179**	0.003
		G_{3A}	41	2.2	0.344	0.357	−0.037	0.542
	2	G_1	14	2.2	0.352	0.260	**0.261**	0.001
		G_2	33	2.3	0.338	0.285	**0.159**	0.010
		G_{3A}	17	2.3	0.374	0.305	**0.172**	0.022
	3	G_1	16	2.2	0.328	0.280	**0.140**	0.011
		G_2	37	2.2	0.336	0.254	**0.248**	<0.001
	4	G_{3S}	20	2.1	0.312	0.299	0.045	0.309
		G_{3A}	20	2.2	0.313	0.236	**0.239**	0.001
	5	G_1	12	2.2	0.373	0.250	**0.327**	0.001
		G_2	34	2.3	0.342	0.275	**0.200**	<0.001
		G_{3A}	20	2.2	0.369	0.317	**0.149**	0.008

HWE test *P* value with H_I heterozygote deficiency (significant F_{IS} values in bold)

n sample size, *Ar* mean allelic richness, *Hs* and *Ho* expected and observed heterozygosities

significantly different from zero in autumn samples. Conversely, population genetic differentiation increased from G_1 to G_3, and was significant in G_2 and G_3, despite generally low values. Population selfing rates estimated with RMES did not differ from zero (results not shown), except in G_{3A} of one control sample (control 1) ($s = 0.453$, SD = 0.144, $P = 0.015$), in which global inbreeding was also highest ($F_{IS} = 0.403$). The expected inbreeding due to

Table 4 Parameters of genetic structure estimated per generation or sampling campaign

Generation	F (F_{IT})	θ (F_{ST})	f (F_{IS})
G_1	**0.222** [0.028; 0.491]	0.009 [−0.013; 0.036]	**0.215** [0.031; 0.474]
G_2	**0.255** [0.067; 0.505]	**0.018** [0.002; 0.038]	**0.241** [0.055; 0.489]
G_{3S}	**0.206** [0.062; 0.412]	**0.060** [0.032; 0.102]	**0.156** [0.001; 0.377]
G_{3A}	**0.212** [0.038; 0.411]	**0.057** [0.026; 0.083]	0.164 [−0.008; 0.367]

F, θ, and f are Weir and Cockerham (1984) estimators of Wright's F statistics. Bold values indicate significant difference from zero. Confidence intervals (95 %) based on 1000 btp

Table 5 Comparison of population genetic parameters among control, low input and conventional pesticide treatment groups, as calculated per generation or sampling campaign

Population group	Allelic richness	*Ho*	*Hs*	F_{IS}	F_{ST}
G_1					
Control	2.0	0.272	0.347	0.216	0.006
Low input	2.0	0.278	0.338	0.178	0.029
Conventional	2.0	0.266	0.354	0.249	−0.004
P value	0.804	0.923	0.957	0.651	0.521
G_2					
Control	2.2	0.247	0.359	0.310	0.032
Low input	2.2	0.300	0.369	0.187	0.002
Conventional	2.3	0.271	0.340	0.203	0.015
P value	0.516	**0.005**	**0.005**	**0.043**	0.293
G_{3S}					
Control	2.1	0.315	0.365	0.137	0.034
Low input	2.0	0.304	0.366	0.171	0.046
Conventional	1.9	0.265	0.304	0.131	0.166
P value	**0.006**	0.223	**0.013**	0.878	**0.049**
G_{3A}					
Control	2.0	0.245	0.324	0.244	0.030
Low input	2.1	0.295	0.361	0.184	0.035
Conventional	2.1	0.316	0.348	0.092	0.085
P value	0.166	0.171	0.252	0.459	0.358

P values are based on 1000 permutations of whole samples among the three groups. Bold values indicate statistical significance. Pairwise test are illustrated on Fig. 2

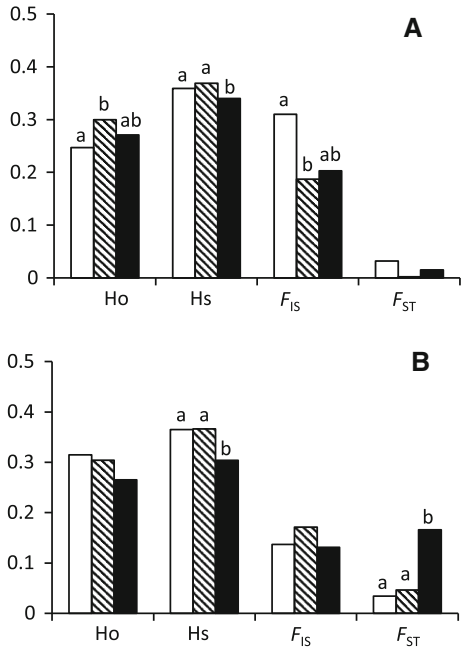

Fig. 1 Comparison of population genetic parameters across pesticide treatment programmes, in G_2 (**a**) and G_{3S} (**b**) samples. *White bars* indicate control populations, *dashed bars* "low input" populations, and *black bars* "conventional" populations. *Letters above bars* denote homogeneous groups inferred from pairwise comparisons based on 1000 permutations of populations among groups. Mean allelic richness (not represented on Fig 1 due to different scale) was significantly reduced in G_{3S} samples collected from "conventional" populations (Ar = 1.93), compared to "low input" (2.05) and control samples (2.06) ($P = 0.006$)

selfing only can be approximated by (S/(2-S), which leads here to a F_{IS} value of 0.293. Hence, from this approximation, selfing would represent about 70 % of the total observed inbreeding in control 1 G_{3A}.

When population parameters were compared according to chemical treatment levels, significant differences were detected in G_2 and G_{3S} samples (Table 5; Fig. 1). Based on the same permutation procedure, pairwise tests showed that in the G_2, *Hs* was significantly higher in populations submitted to low input than to conventional treatments, *Ho* was higher in low input than in control populations, and F_{IS} was higher in control than in low input populations. In the spring sampling of G_3, patterns of variation opposed conventional populations on the one side to low input and control populations on the other: mean allelic richness and *Hs* were lower and genetic differentiation (F_{ST}) was higher in the former group of populations (Fig. 1).

$L_D N_E$ failed to provide accurate estimations of *Ne* (see confidence intervals, supplementary Table S1), whereas *Ne* estimated from the sibship assignment method (SA) presented much lower variance. Based on these estimates, no trend could be detected according to chemical treatments (Fig. 2).

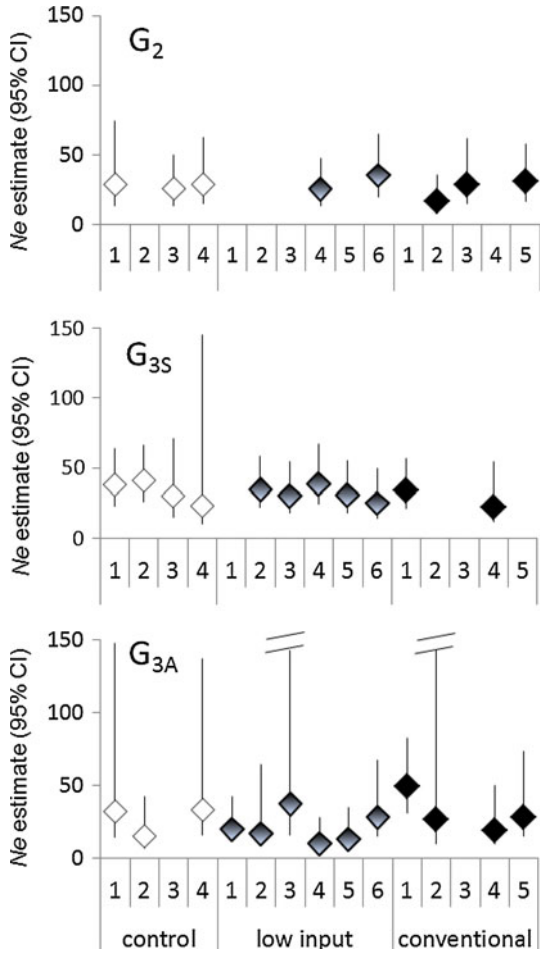

Fig. 2 Graphical representation of *Ne* estimates based on the sibship assignment method (SA; Wang 2009). See also supplementary Table S1 for comparison with estimations based on LD method

Discussion

The present study aimed at estimating short term effects of chronic exposure to pesticides on population genetic parameters of a non-target species. On the basis of 10 polymorphic microsatellite loci, populations exposed for three generations to conventional treatments were found to be less variable and more differentiated than control and low input exposed populations. Otherwise, no difference related to chemical treatments could be detected in inbreeding level, selfing rate, or effective population size.

Effects of pesticide exposure on population genetic parameters

The observed level and distribution of genetic variability were partly consistent with the patterns expected under the study hypothesis, i.e., that chronic pesticide exposure would increase random genetic drift effects, due to increased demographic impact. Previous results on community dynamics emphasized a negative effect of both treatments compared to control conditions, as well as a more pronounced impact of conventional treatment relative to low input treatment on diverse species, including *L. stagnalis* (see b_k-values of the principal response curves, Fig. 3. in Auber et al. 2011). This pattern was only detected in the first year of treatment (November 2008 to Summer 2009), whereas no differences were observed between treatments from Autumn 2009 to Autumn 2010 (Auber 2011). In *L. stagnalis*, individuals exposed to conventional treatment were found to have grown more than their low input and control counterparts, after 10 months spent outdoor (including 5 months of exposure to pesticides), and post-exposure fecundity (as measured under laboratory conditions) was higher in previously exposed snails (conventional and low input) than in control ones (Coutellec et al. 2011). While these traits are not easily interpreted in terms of population size and dynamics, it might be suggested that on the one hand, higher body growth was related to the limitation of intra and interspecific competitors for trophic resources in the course of the experiment (e.g., arthropods were particularly sensitive to insecticides), and that on the other hand, higher fecundity in contaminated snails was also related to higher in situ availability of trophic resources and/or was triggered in response to population size reduction. Thus, indirect effects are likely to have been induced by pesticide treatments, both at the community level, as previously demonstrated in this species (Arts et al. 2006; Caquet et al. 2007), and at the population level through density-dependent processes, as also shown in other invertebrates (Beketov and Liess 2005).

Because the magnitude of random genetic drift is inversely related to population size, we consistently expected random drift effects to increase in the following order: control < low input < conventional treatments, or at least control and low input < conventional. Patterns most consistent with this hypothesis were obtained in the G_3 generation, spring samples (G_{3S}): (1) mean allelic richness and genetic diversity were significantly lower in conventional populations than in control and low input ones; (2) conventional populations were significantly more differentiated that the other ones (F_{ST} values). The lack of significant difference between low input and control populations is likely to result from the lighter impact of this programme on *L. stagnalis* abundance, and also from low resolution associated with the level of standing genetic variation at the start of the experiment (maximum allelic richness: 2.5).

Discrepancies were observed between spring and autumn samples of the same generation, since in the latter group of samples, no difference could be detected between chemical treatments. Sample size might be responsible for

this lack of consistency, as autumn samples were generally smaller than spring ones (see Table 1). However, as sample size reflected our ability to collect snails and thus indirectly population size at the time of sampling, we might have expected random drift effects to be more evident in autumn. This result emphasizes the limit of the method when genetic variability is low. It is to be recalled that low sample size increases the rate of false negative (type II error), due to weak statistical power. In the present study, mean sample size was 28.3 ± 2.1 individuals over all generations and populations, and 32.3 ± 2.3 in the subgroup of outdoor-born cohorts, i.e., values commonly applied in population genetics studies. However, G_1 and G_{3A} cohorts were characterized by particularly low sample sizes, and parameter comparisons were thus expected to suffer from low statistical power in these cohorts. Among G_1 samples, we did not expect any genetic pattern or effect, since these represented the founding generation, which originated from the same set of families introduced in each mesocosm. Conversely, G_{3A} snails were born in mesocosms, and at the time scale of the study, we expected to detect genetic effects of treatments (if occurring) primarily among these samples. Therefore, as already mentioned, the lack of clear pattern from analyses performed on G_{3A} is probably the result of small sample size and weak statistical power.

Comparatively, G_{3S} individuals were more numerous, and patterns inferred from this cohort were indeed in line with the tested hypothesis (lower variability and higher differentiation of populations submitted to conventional treatments).

In the G_2, *Hs*, *Ho* and F_{IS} exhibited patterns that could not be interpreted in terms of chemical pressure. Indeed, in this generation, although *Hs* was higher in low input populations than in conventional ones, control populations were intermediate. Similarly, low input populations had higher observed diversity (*Ho*) and lower inbreeding coefficient than control ones, but conventional populations were intermediate. A possible explanation for this pattern is that one generation was not sufficient to detect random drift effects, in accordance with low levels of F_{ST} values in the G_2, whatever the treatment. However, significant differences occurred, which remain difficult to interpret.

Effective population size was estimated using two independent methods, one based on linkage disequilibrium, the other on sibship assignment. While none of them detected any difference between treatments (when other parameters did), the latter provided estimates with much lower confidence intervals, and seems thus to be more reliable. *Ne* is known to be very difficult to estimate and a number of methods have been proposed in the literature (see Wang 2005). Our results clearly show that, although problems known to affect *Ne* estimation such as generation overlap or gene flow (Wang and Whitlock 2003) were not present, the scale of the study and the level of standing genetic variability were not suited to assess differences in this parameter, which should be interpreted cautiously.

Timing of effects

The present study was performed under an extremely short term scale (3 generations). The design may thus be questionable, for population genetic parameters may not reflect what happened two generations back. It is to be noted that our design started with almost exactly the same gene pool and structure in each population. Before treatments, all established populations could thus be considered as replicate populations and confounding factors were not expected to mask the effects of pesticide exposure. In a previous study also based on experimental populations of *L. stagnalis*, we were able to detect local drift load on a similar timescale, through heterosis among a set of initially small populations, as compared to larger ones (Coutellec and Caquet 2011). Thus, although we acknowledge that the power of our design was weak in terms of number of generations, it showed that random genetic drift may leave detectable traces on a very short timescale, in accordance with the hypothesis that evolutionary impacts may occur very rapidly under stress (see Hoffmann and Hercus 2000). It should also be noted that short but drastic bottlenecks have been shown to have more profound consequences on population genetic parameters such as allelic richness or expected heterozygosity, than long and diffuse ones (England et al. 2003).

Initial population characteristics

Forty isofemale lines were used to set up the founding generation, which stemmed from a unique population. This population is also at the origin of the laboratory culture RENILYS® maintained by the U3E. The set of microsatellite markers used were selected as being polymorphic in this original population. The level of genetic diversity was weak in this culture, as compared to other laboratory cultures or natural populations (see Besnard et al. 2013; Kopp et al. 2012). Consistently, low levels of variability were generally observed in the present study, which limited the resolution of population structure. Moreover, significant F_{IS} values were observed in all G_1 samples (founders that were retrieved in spring 2009), except in one mesocosm (low input 4). This result strongly suggests that isofemale lines set up from wild-caught adults (G_0) originated in large part from self-fertilization. Indeed, G_0 snails had been collected in November 2007, i.e., clearly after the end of the reproductive period, and isolated in the laboratory. Half of the snails laid their first clutches more than a month after

isolation, despite favorable conditions (unpublished data). We hypothesize that putative allosperm reserves (see Jarne et al. 2010) were mostly exhausted at the time of capture, and that under favorable laboratory conditions (food ad libitum, Temp. 20 °C and photoperiod 12D:12L, as compared to outdoor conditions in November 2007), snails reactivated reproduction via enforced self-fertilization (as they were not allowed to copulate in the laboratory). This is consistent with the fact that significant inter-family variation was observed among lines for a number of life history traits under standard laboratory conditions (Coutellec et al. 2011). Indeed, higher divergence is in theory expected between different full-sib families than among half-sib ones if the latter group share some degree of common paternity.

Selfing versus biparental inbreeding

Analysis of the mating system, performed using a method independent of F_{IS} (RMES, David et al. 2007), revealed that self-fertilization did not contribute to the observed levels of inbreeding, except in one case, a control population sample, in which F_{IS} was also highest. In all other populations, inbreeding when significant was contributed by biparental inbreeding only. Hence, the hypothesis that stressful conditions associated with pesticide exposure might have induced the expression of self-fertilization was not confirmed in the present study. Biparental inbreeding was present in almost all populations, suggesting that initial census population size (40 unrelated adults) typically led to mating among relatives in subsequent generations. This pattern is consistent with the weak proportion of G_1 snails retrieved after 10 months (marked individuals, 20 % mean recapture rate), if less than ten snails on average per population effectively contributed to the G_2.

In conclusion, our experiment suggests that random genetic drift may be rapidly inflated by chronic exposure to multiple pesticides, as reflected by the significant decrease in genetic variability and increase in genetic differentiation in the group of demographically most impaired populations (conventional treatment). It points to the importance of stochastic processes resulting from such exposure conditions, which should not be neglected, relative to expected adaptive responses. Small populations are expected to have lower ability to respond to environmental change and to selective forces, because of genetic variability loss, and also because selection is less efficient (see "Introduction"). Finally, as the accumulation of slightly deleterious mutations may lead such populations to extinction, random genetic drift clearly deserves specific attention in ecological risk assessment.

However, the study was limited by both the short timescale and the low level of genetic variability initially available. We suggest that population genetic parameters should be used with serious caution, and that, basically, population replication should be accounted for as much as possible in any design aimed at testing the past or current influence of pollutants on the genetic structure of natural populations.

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