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Heterogeneity of selection and the evolution of resistance

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1 Heterogeneity of selection and the evolution of resistance

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24 **The evolution of resistance to pesticides and drugs in pests and pathogens is a textbook**
25 **example of adaptation to environmental changes and a major issue in both public health**
26 **and agronomy. Surprisingly, there is little consensus on how to combine selection**
27 **pressures (i.e. molecules used in the treatment of pests or pathogens) over space and time**
28 **to delay or prevent this evolutionary process. By reviewing theoretical models and**
29 **experimental studies, we show that higher levels of heterogeneity of selection are**
30 **associated with longer-term sustainability of pest or pathogen control. The combination**
31 **of molecules usually outcompetes other resistance management strategies such as**
32 **responsive alternation, periodic application or mosaic, because it ensures “multiple**
33 **intra-generational killing”. A strategic deployment over space and/or time of several**
34 **combinations can ensure “multiple inter-generational killing”, further delaying the**
35 **evolution of resistance.**

36

37 **The worrying issue of the evolution of resistance**

38 Throughout history, humans have used a variety of strategies to control diseases and their
39 vectors as well as pests impacting crops and domestic animals. As far back as the eighth
40 century BC Homer refers to the use of sulfur to fumigate homes. Arsenic, an insecticide
41 recommended by the Roman naturalist Pliny the Elder in the first century, was used in the
42 tenth century by the Chinese to control garden pests. From the 1940s, the discovery of modern
43 pesticides – such as DDT – and of most of the major classes of antibiotics appeared to offer

4 3

5

44 the key to controlling pests and pathogens. Most of these measures were relatively cheap and
45 ensured very high levels of control. During two following decades those molecules have been
46 widely used in fields, farms, homes and hospitals to treat crops, animals and humans saving
47 yields and lives. Unfortunately, one of the drawbacks of these treatments for killing pests and
48 pathogens is that they exert selection pressures on target populations, leading to the evolution
49 of resistance mechanisms reducing the efficacy of treatments (for insecticides [1], for
50 herbicides [2], for antibiotics [3, 4], for HIV-1 protease inhibitors [5]).

51 The evolution of resistance to pesticides and drugs has offered several case studies of
52 adaptive evolution and can be considered a valuable example for other evolutionary changes
53 more difficult to perceive and analyze. Hence, studies of the evolution of resistance to various
54 pesticides have improved our understanding of the molecular mechanisms involved in
55 adaptation [6] and dominance [7], the epistatic relationships between loci [8] and the fitness
56 costs of adaptive mutations[9].

57 The evolution of resistance to pesticides and drugs is not only a textbook example of
58 adaptation. It is, above all, a major issue for both public health and agronomy, because the
59 number of drugs and pesticides with different mechanisms of toxicity and acting on
60 independent targets has proved to be limited (see e.g. for antibiotics [10] and for pesticides
61 [11]) (Box 1). Only a few new active molecules have been discovered in the last 30 years. A
62 new wave of R&D on drugs and pesticides, with the exception of that relating to insecticidal
63 toxins, would be unlikely to yield substantial public health and crop protection options within
64 the next 10 to 15 years [12]. In the meantime, we need to protect the existing molecules.

65 Fortunately, most classes of pesticides and antibiotics [10] include several molecules that are
66 still active and for which, at least in some cases, there is still no sign of resistance. This raises
67 questions about how we can combine these molecules over time and space to preserve their
68 efficacy for as long as possible.

69 In the literature, four principal basic strategies combining two (or more) molecules over
70 time and/or space have been considered, to delay the evolution of resistance to drugs and
71 pesticides: *Responsive alternation*, *Periodic application*, *Mosaic* and *Combination* (Box 2). Is
72 one particular strategy intrinsically better than the others? Conversely, does the ranking of
73 strategies depend on the target organism or the pesticide or drug considered? Theoretical
74 models predicting the outcome of selection pressures and experimental selection on pests and
75 pathogens can be used to test such predictions. Here, we review the results obtained with
76 theoretical models and in empirical studies for various pesticides and drugs (generally
77 considered separately, by ecologists and agronomists on the one hand and medical scientists
78 on the other [13, 14]). We show that some consensus can be reached on the deployment of
79 selection pressures over time and space to delay or prevent the evolution of resistance in pest
80 and pathogen populations

81

82 **Theoretical comparisons between strategies**

83 We searched for articles that explicitly compared, in the same study, at least two of the four
84 strategies — *Responsive alternation*, *Periodic application*, *Mosaic* and *Combination* (whether
85 *half-* or *full-dose*, see Box 2) — in terms of their efficacy for delaying the evolution of

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86 resistance to more than one pesticide or drug. We therefore excluded all studies that
87 considered several molecules but modeled the evolution of resistance to only one molecule. A
88 search of the Resistance to Xenobiotic (REX) bibliographic database [13, 14] for articles
89 relating to the modeling of resistance evolution identified 20 relevant articles. Further
90 searches in the Web of Science and Google Scholar and screening of the articles cited in the
91 initial 20 articles yielded an additional nine articles. Half of those articles were related to
92 either insecticide or antibiotic resistance.

93 Based on the 29 articles retained (Table S1), we identified a clear ranking of the strategies
94 in terms of their efficacy for delaying resistance: *Combination* > *Periodic application* =
95 *Mosaic* > *Responsive alternation* (Table 1). *Combination* was at least as good as, or
96 outperformed *Responsive alternation*, *Periodic application* and *Mosaic* in more than 80% of
97 the comparisons. *Half-dose Combination* was found to have been little studied and
98 comparisons of *Combination* with other strategies were somewhat biased because a *full-dose*
99 *Combination*, by doubling the dose of pesticide or drug used, increases overall selection
100 pressure. *Responsive alternation* was less effective than *Periodic application* and *Mosaic* in all
101 comparisons. The ranking of *Mosaic* and *Periodic application* was, by contrast, not
102 straightforward. These two strategies were compared mostly to determine whether *Periodic*
103 *application* (referred to as *Cycling* in clinical studies, see Box 2) could delay resistance to
104 antibiotics more effectively than *Mosaic* (referred to as *Mixing* in clinical studies, see Box 2)
105 in hospitals or, more specifically, in intensive care units. All the epidemiological models gave
106 the same answer: *Mosaic* > *Periodic application*. By contrast, Roush [15] and Lenormand &

107 Raymond [16], who modeled the evolution of insecticide resistance, found *Periodic*
108 *application* > *Mosaic*.

109

110 *Combination* and “multiple intra-generational killing” at the individual level

111 *Combination* is very effective due to “multiple intra-generational killing” [17], a key feature
112 that can be explained as follows: if resistance alleles at each of two independent loci are
113 present at very low frequency in the pest or pathogen population, then any given individual is
114 extremely unlikely to carry resistance alleles at both loci. If resistance is recessive, then
115 diploid pests and pathogens are only resistant if they are homozygous for the resistance allele
116 at both loci. When resistance alleles are at low frequencies, this probability is very low. Thus,
117 drug or pesticide *A* can kill individuals that are susceptible to this drug or pesticide, whatever
118 their genotype at the locus conferring resistance to drug or pesticide *B* and *vice versa*. This is
119 described as “multiple intra-generational killing”, because most pest or pathogen individuals
120 are susceptible to both molecules and are therefore “killed twice” (Figure 1).

121 The superiority of *Combination* over the other strategies appears to be very robust: in most
122 models, this approach was effective for longer even if input and output parameters were
123 varied. Its comparative advantage is particularly high when: (1) resistance to each pesticide or
124 drug is initially rare [17-21], (2) resistance to each pesticide or drug in the combination are
125 controlled by independent loci (no cross-resistance) [17, 22-24], (3) there is a high rate of
126 recombination between the loci [17, 20, 23, 24], (4) in diploids, homozygous susceptible
127 individuals have a high mortality [15, 21], (5) in diploids, resistance to each pesticide is

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128 functionally recessive [17, 23, 25-27], (6) the pesticides or drugs are of similar persistence [15,
129 27] and (7) some of the population remains untreated [20, 22, 24, 25]. Even if these
130 conditions are not completely met, *Combination* appears at least as good as the three other
131 strategies.

132

133 *Degree of treatment heterogeneity and “multiple killing”*

134 The most recent approaches in medicine focus on antibiotic heterogeneity [28, 29], the idea
135 being that higher degree of treatment heterogeneity (*DTH*) are associated with a slower
136 evolution of resistance. Mani [30] explored this idea for insecticide resistance, more than 20
137 years ago. He showed that, after *Combination*, the most promising strategy was not to vary
138 applications of a given molecule over time (*Periodic application*) or space (*Mosaic*), but to
139 alternate the insecticides used over *both* time and space, thereby maximizing the *DTH*.

140 To our knowledge, the relationship between *DTH*, temporal or spatial selection
141 heterogeneity, and the sustainability of efficacy for a given molecule has never been clearly
142 formalized. We suggest that *DTH* should be defined as the probability that a set of resistance
143 genes is confronted to more than one pesticide or drug within or between generations. In case
144 of periodic application, offsprings from individuals resistant to one molecule will be treated
145 with another molecule depending on the generation time of the pathogen or pest and on the
146 period of application of the drug or pesticide. These offspring would be expected to be
147 susceptible to the second molecule, particularly in the absence of cross-resistance and if
148 resistance genes are independent of each other. In this case, *DTH* therefore ensures “multiple

149 inter-generational killing” at the colony or family level (Figure 1), because the first molecule
150 kills most individuals in the parental generation and the second molecule then kills the
151 offspring of the few survivors. As explained before, *Combination* ensures “multiple intra-
152 generational killing” and a maximal *DTH* because every individual suffers simultaneously
153 both molecules. In a *Mosaic* set up the survivors to the first molecule can disperse and then be
154 killed by the second molecule. If dispersal distances are larger than the scale of mosaic unit
155 then *Mosaic* can also lead to “multiple intra-generational killing”.

156 All things being equal, higher *DTH* should be associated with longer-term sustainability of
157 pesticides or drugs. *Responsive alternation* systematically results in the lowest *DTH*, because
158 the offspring are treated with the same molecules as their parents until the population size
159 reaches unacceptable levels. Depending on the pattern of pest or pathogen dispersal, its
160 generation time and the temporal and spatial scales of treatment, higher *DTH* can be achieved
161 with either *Periodic application* or *Mosaic* strategies, or through the use of a combination of
162 these two extreme strategies.

163

164 **Empirical comparisons between strategies**

165 In 1983, Georghiou [31] stated that: “*Perusal of pertinent literature reveals that there are*
166 *more papers discussing the value of mixture [i.e. combination] (as well as rotation [i.e.*
167 *periodic application]) than those that report actual research on the subject”*. Unfortunately,
168 this remains true in 2012. Using the Web of Science, Google Scholar and the references cited
169 in recent articles on this topic, we found only 17 empirical studies – half of them being on

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170 insecticide resistance – comparing at least two strategies in laboratory, greenhouse or field
171 conditions (Table S2).

172 In the 17 empirical studies identified, the ranking of efficacy was *Combination = Periodic*
173 *application > Responsive alternation* (Table 1). Indeed, in five of eight comparisons,
174 *Combination* was found to be as good as *Periodic application*, and *Responsive alternation*
175 never outperformed either of these two strategies. It was not possible to rank *Mosaic* reliably,
176 because too few comparisons included this strategy. *Mosaic* outperformed *Periodic*
177 *application* and *Responsive alternation* in two independent studies but was found to be less
178 effective than *Combination*, *Periodic application* and *Responsive alternation* in the other four
179 comparisons (Table 1).

180 Although *Combination* appeared to be the best strategy in theoretical models, it did not
181 clearly outperform *Periodic application* in empirical studies. This discrepancy between
182 theoretical and empirical results can simply reflect time constraints. Indeed, in most
183 experimental studies, treatments were applied during a fixed number of generations. In most
184 cases, resistance emerged when molecules were used singly, but not when they were
185 combined over space and/or time (*Periodic application*, *Mosaic* or *Combination*). Thus,
186 several studies reported an absence of resistance development for at least one molecule for
187 both *Combination* and *Periodic application* strategies [e.g. [32-36]], making it impossible to
188 draw any firm conclusions concerning possible differences in efficacy between these two
189 strategies. The conclusion that these two strategies are similar in efficacy is thus valid for the

190 number of generations over which selection took place, but might not hold absolutely true *per*
191 *se.*

192 The discrepancy between theoretical and empirical results can also result from the use of
193 experimental settings that decrease the advantage of *Combination* over other strategies. As
194 mentioned above, empirical studies are limited by the number of generations that can be run.
195 They are also limited by the number of individuals per generation that can be manipulated.
196 These experimental constraints have two important consequences. First, empirical studies
197 focus on the evolution of resistance alleles already present in populations rather than on
198 resistance alleles acquired *de novo* by mutation or horizontal transfer. Hence, in all but one of
199 the experimental studies (see Table S2), a deliberate decision was taken to have a high
200 frequency (i.e. $> 10^{-3}$) of resistance to at least one pesticide or drug at the start of selection,
201 thereby decreasing the efficacy of *Combination* by violating one of the favoring conditions
202 [17-21]. Second, a sufficiently large number of individuals must survive pesticide or drug
203 treatments to establish the next generation. Consequently, the selection pressure applied in
204 such experiments generally varies between 0.5 and 0.8, corresponding to low doses. In such
205 cases, resistance can be functionally dominant, further decreasing the comparative advantage
206 of *Combination* over the other strategies [17, 23, 25-27]. Experimental settings with high
207 initial frequencies of resistance alleles and low selection pressures can, in some cases,
208 approach real conditions. Molecules newly released onto the market are sometimes used in
209 combination with other molecules for which resistance has already been selected in the
210 targeted pest or pathogen populations, for economic purposes. Selection at low doses can also

211 occur in field conditions because of the dilution of the molecules and their degradation over
212 time.

213 Nevertheless, one specific feature of empirical studies clearly differs from practice. As
214 pointed out above, the presence of untreated individuals from refuges increases the success of
215 *Combination*. However, nine of the 17 empirical studies were conducted without such refuges
216 (Table S2). This is unfortunate, because such refuges could easily be included in studies of the
217 selection of pesticide resistance. Leaving a fraction of the population free of pesticide
218 exposure would have better mimicked the conditions in fields, hospitals and care units.
219 Indeed, a significant proportion of the pests or pathogens often remain untreated
220 unintentionally. Dormant weeds, resting spores of fungi, hidden mosquito breeding sites, soil
221 seed banks or field borders, alternative hosts, or people outside the medical system are very
222 common and constitute unplanned refuges of pest and pathogens.

223

224 **Can *Combination* be outcompeted?**

225 One particular condition can render *Combination* inferior to other strategies. This condition is
226 the occurrence of fitness costs, resulting in resistant individuals being less fit than susceptible
227 individuals in the absence of the pesticide or drug. Such costs might lead to the
228 counterselection of resistance alleles and would therefore delay, if not prevent, the
229 development of resistance. The expression of this cost would require spatial or temporal
230 variation in pesticide or drug selection, with locations or periods of time in which one of the
231 pesticide or drug is absent. *Combination* is the only strategy combining two molecules that

232 does not generate such variation and therefore it is the only strategy that does not allow the
233 expression of a resistance cost. Consequently, fitness costs can facilitate the mitigation of
234 resistance in all strategies except *Combination*. This can explain why Dobson *et al.* [37]
235 (theoretically) and Immajaru *et al.* [38] (experimentally) found *Combination* to be less
236 effective than *Periodic application* and *Mosaic* (see Tables S1 and S2). Indeed, their
237 theoretical and biological models were characterized by very high fitness costs and an absence
238 of refuges (see below).

239 In practice, fitness costs might make *Combination* worse than other strategies only rarely.
240 First, the “multiple intra-generational killing” provided by combination approaches might be
241 sufficient to ensure the superiority of this strategy in many cases, even in the presence of
242 fitness costs. Second, although mutations conferring resistance are often costly (e.g. for
243 herbicides [39], for insecticidal proteins [40], for antibiotics [41], for anti-virus [42]),
244 decreases in fitness can be attenuated or even completely abolished by compensatory
245 mutations (for herbicides [43], for antimicrobial drugs [44], for antibiotics [45], for anti-virus
246 [46]) or through interactions with other resistance mutations [47]. Over time, costly resistance
247 mutations can also be replaced by resistance mutations associated with lower fitness costs
248 [48]. Finally, when part of the population remains untreated, fitness costs counteract the
249 selection of resistance alleles, even for *Combination*. Untreated individuals can be actively
250 preserved by the use or maintenance of refuges for pests and pathogens. The use of refuges is
251 not possible in hospitals, because it would be unethical not to treat infected humans with
252 antibiotics or other drugs. However, the community outside hospitals constitutes a refuge for

253 most pathogen populations and individuals carrying pathogenic bacteria or viruses but
254 displaying no symptoms, or only minor symptoms, are left untreated. Finally, pathogens or
255 pests generally escape treatments even within the host or the field, because treatment coverage
256 is rarely complete.

257

258 **Increasing *Combination's* degree of treatment heterogeneity**

259 The number of molecules that can actually be used in a *Combination* is limited by resistances
260 that have already developed (Box 1) and several challenges underlined in Box 3. Generally,
261 the concomitant use of a large number of molecules entails higher costs, which can outweigh
262 the benefits of delaying or preventing resistance in the eyes of the stakeholders. Thus,
263 combinations containing all the available molecules are unlikely to be used. However, it might
264 be possible to use several different *Combinations* to treat a given pest or pathogen. These
265 combinations would ideally be used so as to ensure the highest *DTH*, yielding “multiple intra-
266 generational killing” (at the individual level) and “multiple inter-generational killing” (at the
267 colony or family level). Depending on the distances over which dispersal occurs, the highest
268 *DTH* can be provided by *Mosaic*, *Periodic application* or a complex temporal and spatial
269 combination of the various combinations.

270 This might have practical consequences. For example, in antibiotic resistance management,
271 treatment heterogeneity is currently defined at the level of the hospital rather than the
272 pathogen. The theoretical and empirical studies reviewed here show that diversity in antibiotic
273 use between care units or beds at a given time (i.e. a *Mosaic* strategy) is more sustainable than

274 cycling different antibiotic regimens over time (i.e. a *Periodic application* strategy). This is
275 because, at the scale relevant to bacterial populations, *Mosaic* imposes greater *DTH* than
276 *Periodic application* [29, 30, 51, 52]. This is particularly true when the cycle of each antibiotic
277 regimen is very long, extending over several months. Indeed, due to its short generation time,
278 a bacterial colony is more likely to encounter the second antibiotic in a *Mosaic* implemented
279 at the scale of the bed or at the scale of the care unit than in a *Periodic application* based on
280 the cycling of antibiotics over several months. Another hypothesis has been put forward by
281 Boni et al [49] to explain the higher performance of *Mosaic* over *Periodic application*:
282 *Periodic application* degrades the mean fitness of the parasite population more quickly than
283 *Mosaic*, making it easier for new resistant types to invade and spread in the population.

284 Although difficult to implement, we suggest that *Periodic application* at the level of the
285 patient, rather than the hospital (or care unit), might result in greater *DTH* than *Mosaic*.
286 Alternating antibiotics to treat patients would increase the likelihood of “multiple inter-
287 generational killing” — i.e. the probability of colonies resistant to a given antibiotic being
288 treated, and therefore killed, by another antibiotic in the next generation.

289

290

291 **Beyond Combination and degree of treatment heterogeneity: protecting populations against**
292 **the emergence of resistance alleles**

293 The question of how to combine pesticides and drugs over time and space is only one part of
294 the overall debate on resistance management. The dose of the molecules used must also be

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295 considered. Resistance management strategies sometimes include the use of high doses of
296 pesticides and drugs. For bacterial and HIV infections, this has been referred to as the “Hit
297 hard and early” approach [50]. Interestingly, different rationales are applied to pesticides and
298 drugs. For drugs, the reason for treating “hard” is to decrease the size of the pathogen
299 population as much as possible, to prevent the appearance of resistance alleles. For pesticides,
300 high-dose strategies are designed not only to avoid the emergence of new resistance alleles
301 [51, 52], but also to avoid the building of polygenic resistance [53] and to make resistance of
302 diploid pests functionally recessive [54]. The use of a high dose can also enlarge the spectrum
303 of pests targeted. This is particularly true for herbicides, as fields generally contain more than
304 one weed species that must be controlled.

305 The drawback of hitting hard is that it increases the costs associated with resistance
306 management (Box 3). This strategy can actually be counterproductive if resistant pathogens
307 are already established [52, 55]. However, in the case of new molecules for which no
308 resistance has been detected, this approach can well be the most appropriate, provided that the
309 costs are sustainable. Unfortunately, most mathematical models of the evolution of pesticide
310 resistance assume that population size is infinite – but see e.g. [53, 56]. Consequently,
311 resistance alleles are generally assumed to be initially present at all resistance loci in the
312 population. In fact, natural populations are limited in size and might contain no resistance
313 alleles. In such cases, the appearance and early increase in frequency of resistance alleles is a
314 stochastic process dependent on the balance between mutation rates and population size. This
315 stochasticity is also largely ignored in empirical studies. As indicated above, empirical studies

316 are always performed at locations or using strains in which resistance to at least one molecule
317 occurs at a relatively high frequency which constitute an unfavorable situation for high-dose
318 strategies.

319 There is therefore a need for both theoretical and empirical studies to further investigate the
320 evolution of resistance in conditions allowing stochastic events [57]. In such situations,
321 'hitting hard' likely results in a greater efficacy of *Combination* than of other strategies and
322 *full-dose Combination* certainly provides populations with the highest level of protection
323 against the emergence of resistance alleles. The use of high-dose *Bt* crops could be seen as a
324 life-size experiment testing this hypothesis. Interestingly, in the US, populations of the pink
325 bollworm, *Pectinophora gossypiella*, targeted by *Bt* cotton, and of the European corn borer,
326 *Ostrinia nubilalis*, targeted by *Bt* maize, have been declining from year to year [58, 59]. For
327 the pink bollworm, sterile moths releases have been successful in suppressing the emergence
328 of resistance alleles to *Bt* cotton [60], a cornerstone for the multi-tactic eradication program of
329 this pest [61]. This provides some hope that pest populations could be eliminated over a wide
330 area before resistance alleles emerge and spread.

331

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336 **Glossary**

337 **Cross-resistance:** a resistance to a pesticide or drug that also confers resistance to another
338 pesticide or drug.

339 **Degree of treatment heterogeneity (DTH):** the probability that a set of resistance genes is
340 confronted to more than one pesticide or drug during a certain amount of time, be it within or
341 between generations.

342 **Insecticidal toxins:** toxins produced by bacteria, mostly *Bacillus thuringiensis* and *B.*
343 *sphaericus*, and used in sprays or in genetically engineered plants to control insects.

344 **Multiple intra-generational killing:** a strategy that consists in using a variety of pesticides or
345 drugs on each pest or pathogen individual, in order to maximize the probability that each
346 individual is killed. An individual resistant to molecule A but susceptible to molecule B will
347 be killed if treated simultaneously by molecules A and B.

348 **Multiple trans-generational killing:** a strategy that consists in using a variety of pesticides
349 or drugs on successive generations of pests or pathogens, in order to maximize the probability
350 that the offspring of resistant individuals is killed. The offspring of an individual resistant to
351 molecule A but susceptible to molecule B will be killed by molecule B.

352 **Recessive resistance allele:** an allele that confers resistance to diploid pests and pathogens
353 only if present in homozygous state. A dominant resistance allele confers resistance when
354 both in heterozygous and homozygous states.

355 **Refuge:** areas, fields or group of pests or pathogens remaining untreated by pesticides or
356 drugs.

357 **Resistance:** a heritable change in a population that is reflected in the ability of individuals to
358 survive and reproduce in the presence of environmental conditions that once killed most
359 individuals of the same species.

360 **Resistance cost:** a negative pleiotropic effect of a resistant genotype that results in a lower
361 fitness of resistant individuals compared to susceptible ones in absence of pesticide or drug.

362 **Resistance gene:** a gene at which one or more alleles confer resistance to pesticides or drugs.

363 **Resistance management strategy:** a strategy devoted to delay or prevent the evolution of
364 resistance in a population of pests or pathogens. *Mosaic, Periodic application, Combination*
365 and *Responsive alternation* (see box 2) are simple resistance management strategies using
366 more than one pesticide or drug.

367 Box 1. The evolution of resistance to pesticides and drugs

368 Almost 8,000 cases of resistance to 300 insecticide compounds have been reported in more
369 than 500 species of arthropods [1] (Arthropod Pesticide Resistance Database (APRD,
370 www.pesticideresistance.com)). Similarly, 300 cases of field resistance to 30 fungicides have
371 been reported in 250 species of phytopathogenic fungi (The Fungicide Resistance Action
372 Committee database (FRAC, <http://www.frac.info>)). The International Survey of Herbicide-
373 Resistant Weeds (ISHRW, www.weedscience.com) has suggested that there are currently
374 about 370 resistant biotypes in 200 weed species in 570,000 fields. The situation is most
375 critical for antibiotic resistance. Genes conferring resistance to antibiotics are ubiquitous in
376 bacteria and highly diverse. The Antibiotic Resistance Genes Database (ARGD,
377 <http://ardb.cbc.umd.edu/>), developed by Liu and Pop [62], lists more than 23,000 potential
378 resistance genes of about 400 types, conferring resistance to 250 antibiotics in 1,700 species
379 of bacteria from 270 genera. Strains from highly pathogenic bacteria, such as *Tuberculosis*
380 *bacilli*, resistant to all known classes of antibiotics have recently been described [63].

381 In addition, most of the major classes of antibiotics were first isolated between 1940 and
382 1960 [64]. The more recently commercialized drugs and pesticides are often variants of
383 previously isolated or synthesized compounds and are therefore not particularly effective
384 against the prevailing resistance mechanisms (see for herbicides [65], for insecticides [66], for
385 antiviral drugs [67] and for antibiotics [68]). The cost of developing new drugs and pesticides
386 has been further increased by the tightening of requirements by regulatory authorities,
387 necessitating a larger number of toxicological, clinical and environmental trials [69]. Hence,

388 according to Larson [70], it currently takes about 10 years and up to US\$1 billion to develop a
389 new antibiotic. Similarly 10 to 12 years are required to develop and launch a new pesticide on
390 the market [71].

391 At the turn of the 21st century, the combination of approaches such as genomics [72],
392 proteomics [73] and metabolomics [74] with target-based high-throughput screening strategies
393 [71, 75] appeared very promising for the discovery of new drugs and pesticides with little or
394 no impact on environment and health. However, these new methods and strategies have proved
395 relatively unsuccessful, both for antibiotics [76] and for pesticides [77]. The situation is very
396 different for insecticidal toxins, mostly proteins from *Bacillus thuringiensis*, whether
397 formulated for application in sprays or produced by transgenic plants. The number of toxins
398 identified is increasing [78] and the populations of most of the pests targeted remain
399 resistance-free ([66], but see [79]).

400 **Box 2. Strategies for combining molecules over time and space**

401 Four principal basic strategies combining two (or more) molecules over time and/or space
 402 have been considered for drugs and pesticides. These strategies differ in the way the pesticides
 403 or drugs are combined. In the *Periodic application* and *Responsive alternation* strategies,
 404 molecule use is uniform over space but heterogeneous over time. *Periodic application* involves
 405 temporal cycles of pesticide or drug application, a strategy first suggested by Coyne [80]. By
 406 contrast, *Responsive alternation* corresponds to successive applications of molecules, but
 407 without a cycle. In this approach, a molecule is used repeatedly until the emergence of
 408 resistance, after which the second molecule is introduced, and so on. *Mosaic* — a strategy
 409 first suggested by Muir [81] — concerns a spatial pattern of application for at least two
 410 molecules. Molecule application remains uniform over time and the spatial distributions of the
 411 molecules used do not overlap. Finally, *Combination* is the concomitant use of two or more
 412 molecules over time and space. *Responsive alternation*, *Periodic application*, *Mosaic* and
 413 *Combination* have been referred to by various names within and between the different classes
 414 of pesticides and drugs, as summarized in the following table.

Strategy	Names used to define strategies			
	Antibiotics or antiviral drugs	Insecticides or Bt toxins	Fungicides	Herbicides
<i>Responsive alternation</i>	sequential use	sequence, sequential use, serial use	–	sequence, threshold strategy
<i>Periodic application</i>	cycling, antibiotic rotation, periodic application,	rotation, alternation,	rotation, alternation,	rotation

<i>n</i>	sequential use	sequential use	sequence	
<i>Mosaic</i>	mixing, 50-50 treatment, antibiotic diversity, multiple first-line therapy	mosaic	–	mosaic
<i>Combination</i>	combination, antibiotic diversity, simultaneous strategy	mixture, pyramiding	mixture, combination	mixture, combination, double knockdown

415

416 In practice, molecules in *Combinations* are combined in variable ratios and at different
 417 doses. Strategies based on both *full-dose* and *half-dose Combinations* have been proposed. In
 418 the *full-dose Combination* strategy, each pesticide or drug is applied at the dose at which it
 419 would be used if applied alone, whereas, in the *half-dose* strategy, the dose of each pesticide
 420 or drug is half that used when the compound is applied alone. Consequently, the final overall
 421 dose of the *full-dose* strategy is equivalent to twice that applied if each molecule were to be
 422 used alone, whereas the final overall dose of the *half-dose Combination* strategy corresponds
 423 to the dose at which each molecule would be applied if used alone.

424 Practical recommendations on the strategy to be used depend on the target organism. For
 425 instance, *Combinations* are currently recommended in the treatment of HIV [82], tuberculosis
 426 [83] and malaria [84]. Pesticides are also increasingly used as combination rather than as
 427 individual compounds, as exemplified by the new generation of *Bt* crops, which produce
 428 several independent toxins against the target pests [85]. However, *Combination* is not the
 429 current *default* strategy in antibiotic treatment, particularly in the community, and is not

430 recommended for several pesticides (e.g. for the control of *Anopheles*, the vector of malaria,

431 [86]).

432 **Box 3. Challenges with *Combinations***

433 Imagine that two molecules are available and that all conditions are satisfied for their
434 combination to outperform all other strategies for delaying the evolution of resistance. Would
435 *Combination* become THE optimal strategy for use with any given set of pesticides and
436 drugs? Probably not! There are several obstacles to the universal recommendation and
437 implementation of this strategy.

438 The possibility of antagonistic effects between molecules – which may seriously reduce
439 pest or pathogen control – constitutes a first obstacle to the use of the *Combination* strategy
440 [87]. Synergistic molecule combinations can be advantageous in controlling pests and
441 pathogens. However, resistance to such combinations can evolve faster than resistance to
442 antagonistic molecule combinations and, in some cases, to individual molecules themselves
443 [88].

444 A second obstacle for using *Combination* is that the molecules prescribed by physicians
445 and used by farmers not only control pests and pathogens, they may also injury crops and have
446 undesirable effects on non-target organisms and human health. The World Health
447 Organization (WHO) has reported that there are about three million human cases of pesticide
448 poisoning annually, resulting in 220,000 deaths worldwide [89], hepatotoxicity, neurotoxicity
449 and lipodystrophy [90-92]. Chemical pesticides have a significant impact on non target plants,
450 fungi and arthropods [93]. Pesticide use can disrupt biological control through direct toxicity
451 [94], indirectly changing the community structure [95] and their predators or parasitoids [96].

452 A trade-off thus exists between controlling the pest with the right dose and limiting the side
453 effects of treatment. Undesirable effects occur when single molecules are used, but they are
454 probably worsened by the use of combinations, because synergy between molecules [97] can
455 increase the threat to the environment [98] and to human health [99].

456 Stakeholders – i.e. companies, users, prescribers and public authorities - diverge on their
457 respective interests, goals and their sensitivity to strategies' costs, depending on the policy
458 implemented. For example, refuges increase the risk of pest/pathogen damage, and in the
459 short term this cost is met directly by users. Similarly, *Combination*, by “multiple intra-
460 generational killing”, can be more efficient for controlling pests/pathogens but, because of the
461 higher dose applied, implies financial costs to farmers or patients (or public authorities if
462 there is social health coverage) as well as increased magnitude of undesirable effects on health
463 and the environment and, thus, the costs to be covered by public authorities.

464 The willingness of the various stakeholders to share the costs depends directly on the
465 extent to which they are likely to be affected by or considered responsible for the emergence
466 of resistance. Hence users are confronted with the so-called “*Tragedy of the Commons*” when
467 exploiting a common property resource [100], even if they are likely to be strongly affected by
468 the evolution of resistance. In most cases, by not playing their part in the management of
469 resistance, each user maximizes their own short-term benefit but favors the selection of
470 resistant pests/pathogens, thus having potentially long-term negative effects for the
471 community.

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684 **Figure Legends**

685 **Figure 1.** Schematic representation of the effect of the different strategies (*Responsive*
686 *alternation, Mosaic, Periodic application* and *Combination*) on the targeted pests or
687 pathogens – here, a mosquito. These strategies can lead to multiple intra-generational killing
688 at the individual level (for *Combination*) or multiple inter-generational killing at the family or
689 colony level (for *Periodic application* and *Mosaic*). This depends on the balance between the
690 spatial and temporal scales of the treatments and the dispersal capacities and generation time
691 of the targeted pests or pathogens. At each generation (G), pests or pathogens are selected by
692 molecule 1, molecule 2 or a combination of these two molecules. Individuals S, R₁ and R₂ are
693 susceptible, resistant to molecule 1 and resistance to molecule 2 respectively. Individuals R₁₂,
694 harboring genes conferring resistance to molecule 1 as well as genes conferring resistance to
695 molecule 2, can survive in a patch treated with a combination of these two molecules. The
696 *Degree of Treatment Heterogeneity (DTH)* defined here as the probability that a set of
697 resistance genes is confronted to more than one pesticide or drug varies among the strategies.
698 *Combination* displays the largest *DTH*, followed by *Periodic applications* and *Mosaic*
699 depending on the generation time, dispersal distance, period and spatial scales of application,
700 and by *Responsive alternation*.

701

702

703 **Table 1.** Side-by-side comparisons of the four strategies in terms of their relative efficacies for delaying or preventing resistance.

Strategies	Theoretical studies	Empirical studies												
		1	2	N ^a	1 > 2	1 = 2	1 < 2	Conditional ^b	N	1 > 2	1 = 2	1 < 2		

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<i>Mosaic</i>	<i>Responsive</i>	3	2	1	0	0	2	1	0	1
<i>alternation</i>										

704 ^a N = number of comparisons in all theoretical and empirical studies

705 ^b The ranking of the strategies depends on the setting for one or several input or output parameters.