Heterogeneity of selection and the evolution of resistance

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Heterogeneity of selection and the evolution of resistance

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

The worrying issue of the evolution of resistance

Throughout history, humans have used a variety of strategies to control diseases and their vectors as well as pests impacting crops and domestic animals. As far back as the eighth century BC Homer refers to the use of sulfur to fumigate homes. Arsenic, an insecticide recommended by the Roman naturalist Pliny the Elder in the first century, was used in the tenth century by the Chinese to control garden pests. From the 1940s, the discovery of modern pesticides – such as DDT – and of most of the major classes of antibiotics appeared to offer...
44 the key to controlling pests and pathogens. Most of these measures were relatively cheap and ensured very high levels of control. During two following decades those molecules have been widely used in fields, farms, homes and hospitals to treat crops, animals and humans saving yields and lives. Unfortunately, one of the drawbacks of these treatments for killing pests and pathogens is that they exert selection pressures on target populations, leading to the evolution of resistance mechanisms reducing the efficacy of treatments (for insecticides [1], for herbicides [2], for antibiotics [3, 4], for HIV-1 protease inhibitors [5]).

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

The evolution of resistance to pesticides and drugs is not only a textbook example of adaptation. It is, above all, a major issue for both public health and agronomy, because the number of drugs and pesticides with different mechanisms of toxicity and acting on independent targets has proved to be limited (see e.g. for antibiotics [10] and for pesticides [11]) (Box 1). Only a few new active molecules have been discovered in the last 30 years. A new wave of R&D on drugs and pesticides, with the exception of that relating to insecticidal toxins, would be unlikely to yield substantial public health and crop protection options within the next 10 to 15 years [12]. In the meantime, we need to protect the existing molecules.
Fortunately, most classes of pesticides and antibiotics [10] include several molecules that are still active and for which, at least in some cases, there is still no sign of resistance. This raises questions about how we can combine these molecules over time and space to preserve their efficacy for as long as possible.

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

Theoretical comparisons between strategies

We searched for articles that explicitly compared, in the same study, at least two of the four strategies — Responsive alternation, Periodic application, Mosaic and Combination (whether half- or full-dose, see Box 2) — in terms of their efficacy for delaying the evolution of resistance.
resistance to more than one pesticide or drug. We therefore excluded all studies that considered several molecules but modeled the evolution of resistance to only one molecule. A search of the Resistance to Xenobiotic (REX) bibliographic database [13, 14] for articles relating to the modeling of resistance evolution identified 20 relevant articles. Further searches in the Web of Science and Google Scholar and screening of the articles cited in the initial 20 articles yielded an additional nine articles. Half of those articles were related to either insecticide or antibiotic resistance.

Based on the 29 articles retained (Table S1), we identified a clear ranking of the strategies in terms of their efficacy for delaying resistance: Combination > Periodic application = Mosaic > Responsive alternation (Table 1). Combination was at least as good as, or outperformed Responsive alternation, Periodic application and Mosaic in more than 80% of the comparisons. Half-dose Combination was found to have been little studied and comparisons of Combination with other strategies were somewhat biased because a full-dose Combination, by doubling the dose of pesticide or drug used, increases overall selection pressure. Responsive alternation was less effective than Periodic application and Mosaic in all comparisons. The ranking of Mosaic and Periodic application was, by contrast, not straightforward. These two strategies were compared mostly to determine whether Periodic application (referred to as Cycling in clinical studies, see Box 2) could delay resistance to antibiotics more effectively than Mosaic (referred to as Mixing in clinical studies, see Box 2) in hospitals or, more specifically, in intensive care units. All the epidemiological models gave the same answer: Mosaic > Periodic application. By contrast, Roush [15] and Lenormand &
Raymond [16], who modeled the evolution of insecticide resistance, found periodic application > Mosaic.

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

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

The superiority of Combination over the other strategies appears to be very robust: in most models, this approach was effective for longer even if input and output parameters were varied. Its comparative advantage is particularly high when: (1) resistance to each pesticide or drug is initially rare [17-21], (2) resistance to each pesticide or drug in the combination are controlled by independent loci (no cross-resistance) [17, 22-24], (3) there is a high rate of recombination between the loci [17, 20, 23, 24], (4) in diploids, homozygous susceptible individuals have a high mortality [15, 21], (5) in diploids, resistance to each pesticide is
functionally recessive [17, 23, 25-27], (6) the pesticides or drugs are of similar persistence [15, 27] and (7) some of the population remains untreated [20, 22, 24, 25]. Even if these conditions are not completely met, *Combination* appears at least as good as the three other strategies.

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

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

To our knowledge, the relationship between *DTH*, temporal or spatial selection heterogeneity, and the sustainability of efficacy for a given molecule has never been clearly formalized. We suggest that *DTH* should be defined as the probability that a set of resistance genes is confronted to more than one pesticide or drug within or between generations. In case of periodic application, offsprings from individuals resistant to one molecule will be treated with another molecule depending on the generation time of the pathogen or pest and on the period of application of the drug or pesticide. These offspring would be expected to be susceptible to the second molecule, particularly in the absence of cross-resistance and if 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
157 All things being equal, higher DTH should be associated with longer-term sustainability of
158 pesticides or drugs. Responsive alternation systematically results in the lowest DTH, because
159 the offspring are treated with the same molecules as their parents until the population size
160 reaches unacceptable levels. Depending on the pattern of pest or pathogen dispersal, its
161 generation time and the temporal and spatial scales of treatment, higher DTH can be achieved
162 with either Periodic application or Mosaic strategies, or through the use of a combination of
163 these two extreme strategies.
164
165 Empirical comparisons between strategies
166 In 1983, Georghiou [31] stated that: “Perusal of pertinent literature reveals that there are
167 more papers discussing the value of mixture [i.e. combination] (as well as rotation [i.e.
168 periodic application]) than those that report actual research on the subject”. Unfortunately,
169 this remains true in 2012. Using the Web of Science, Google Scholar and the references cited
170 in recent articles on this topic, we found only 17 empirical studies – half of them being on
insecticide resistance – comparing at least two strategies in laboratory, greenhouse or field conditions (Table S2).

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

Although Combination appeared to be the best strategy in theoretical models, it did not clearly outperform Periodic application in empirical studies. This discrepancy between theoretical and empirical results can simply reflect time constraints. Indeed, in most experimental studies, treatments were applied during a fixed number of generations. In most cases, resistance emerged when molecules were used singly, but not when they were combined over space and/or time (Periodic application, Mosaic or Combination). Thus, several studies reported an absence of resistance development for at least one molecule for both Combination and Periodic application strategies [e.g. [32-36]], making it impossible to draw any firm conclusions concerning possible differences in efficacy between these two strategies. The conclusion that these two strategies are similar in efficacy is thus valid for the
The discrepancy between theoretical and empirical results can also result from the use of experimental settings that decrease the advantage of Combination over other strategies. As mentioned above, empirical studies are limited by the number of generations that can be run. They are also limited by the number of individuals per generation that can be manipulated. These experimental constraints have two important consequences. First, empirical studies focus on the evolution of resistance alleles already present in populations rather than on resistance alleles acquired de novo by mutation or horizontal transfer. Hence, in all but one of the experimental studies (see Table S2), a deliberate decision was taken to have a high frequency (i.e. > 10^{-3}) of resistance to at least one pesticide or drug at the start of selection, thereby decreasing the efficacy of Combination by violating one of the favoring conditions [17-21]. Second, a sufficiently large number of individuals must survive pesticide or drug treatments to establish the next generation. Consequently, the selection pressure applied in such experiments generally varies between 0.5 and 0.8, corresponding to low doses. In such cases, resistance can be functionally dominant, further decreasing the comparative advantage of Combination over the other strategies [17, 23, 25-27]. Experimental settings with high initial frequencies of resistance alleles and low selection pressures can, in some cases, approach real conditions. Molecules newly released onto the market are sometimes used in combination with other molecules for which resistance has already been selected in the targeted pest or pathogen populations, for economic purposes. Selection at low doses can also
occur in field conditions because of the dilution of the molecules and their degradation over time.

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

**Can Combination be outcompeted?**

One particular condition can render Combination inferior to other strategies. This condition is the occurrence of fitness costs, resulting in resistant individuals being less fit than susceptible individuals in the absence of the pesticide or drug. Such costs might lead to the counterselection of resistance alleles and would therefore delay, if not prevent, the development of resistance. The expression of this cost would require spatial or temporal variation in pesticide or drug selection, with locations or periods of time in which one of the pesticide or drug is absent. Combination is the only strategy combining two molecules that
does not generate such variation and therefore it is the only strategy that does not allow the
expression of a resistance cost. Consequently, fitness costs can facilitate the mitigation of
resistance in all strategies except Combination. This can explain why Dobson et al. [37]
(theoretically) and Immajaru et al. [38] (experimentally) found Combination to be less
effective than Periodic application and Mosaic (see Tables S1 and S2). Indeed, their
theoretical and biological models were characterized by very high fitness costs and an absence
of refuges (see below).

In practice, fitness costs might make Combination worse than other strategies only rarely.
First, the “multiple intra-generational killing” provided by combination approaches might be
sufficient to ensure the superiority of this strategy in many cases, even in the presence of
fitness costs. Second, although mutations conferring resistance are often costly (e.g. for
herbicides [39], for insecticidal proteins [40], for antibiotics [41], for anti-virus [42]),
decreases in fitness can be attenuated or even completely abolished by compensatory
mutations (for herbicides [43], for antimicrobial drugs [44], for antibiotics [45], for anti-virus
[46]) or through interactions with other resistance mutations [47]. Over time, costly resistance
mutations can also be replaced by resistance mutations associated with lower fitness costs
[48]. Finally, when part of the population remains untreated, fitness costs counteract the
selection of resistance alleles, even for Combination. Untreated individuals can be actively
preserved by the use or maintenance of refuges for pests and pathogens. The use of refuges is
not possible in hospitals, because it would be unethical not to treat infected humans with
antibiotics or other drugs. However, the community outside hospitals constitutes a refuge for
most pathogen populations and individuals carrying pathogenic bacteria or viruses but
displaying no symptoms, or only minor symptoms, are left untreated. Finally, pathogens or
pests generally escape treatments even within the host or the field, because treatment coverage
is rarely complete.

Increasing Combination’s degree of treatment heterogeneity

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

This might have practical consequences. For example, in antibiotic resistance management,
treatment heterogeneity is currently defined at the level of the hospital rather than the
pathogen. The theoretical and empirical studies reviewed here show that diversity in antibiotic
use between care units or beds at a given time (i.e. a Mosaic strategy) is more sustainable than
cycling different antibiotic regimens over time (i.e. a Periodic application strategy). This is because, at the scale relevant to bacterial populations, Mosaic imposes greater DTH than Periodic application [29, 30, 51, 52]. This is particularly true when the cycle of each antibiotic regimen is very long, extending over several months. Indeed, due to its short generation time, a bacterial colony is more likely to encounter the second antibiotic in a Mosaic implemented at the scale of the bed or at the scale of the care unit than in a Periodic application based on the cycling of antibiotics over several months. Another hypothesis has been put forward by Boni et al [49] to explain the higher performance of Mosaic over Periodic application: Periodic application degrades the mean fitness of the parasite population more quickly than Mosaic, making it easier for new resistant types to invade and spread in the population.

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

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

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

The drawback of hitting hard is that it increases the costs associated with resistance management (Box 3). This strategy can actually be counterproductive if resistant pathogens are already established [52, 55]. However, in the case of new molecules for which no resistance has been detected, this approach can well be the most appropriate, provided that the costs are sustainable. Unfortunately, most mathematical models of the evolution of pesticide resistance assume that population size is infinite – but see e.g. [53, 56]. Consequently, resistance alleles are generally assumed to be initially present at all resistance loci in the population. In fact, natural populations are limited in size and might contain no resistance alleles. In such cases, the appearance and early increase in frequency of resistance alleles is a stochastic process dependent on the balance between mutation rates and population size. This stochasticity is also largely ignored in empirical studies. As indicated above, empirical studies
are always performed at locations or using strains in which resistance to at least one molecule occurs at a relatively high frequency which constitute an unfavorable situation for high-dose strategies.

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

**Acknowledgments**

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Glossary

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

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

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

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

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

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

Refuge: areas, fields or group of pests or pathogens remaining untreated by pesticides or drugs.
Resistance: a heritable change in a population that is reflected in the ability of individuals to survive and reproduce in the presence of environmental conditions that once killed most individuals of the same species.

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

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

Resistance management strategy: a strategy devoted to delay or prevent the evolution of resistance in a population of pests or pathogens. Mosaic, Periodic application, Combination and Responsive alternation (see box 2) are simple resistance management strategies using more than one pesticide or drug.
Box 1. The evolution of resistance to pesticides and drugs

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

In addition, most of the major classes of antibiotics were first isolated between 1940 and 1960 [64]. The more recently commercialized drugs and pesticides are often variants of previously isolated or synthesized compounds and are therefore not particularly effective against the prevailing resistance mechanisms (see for herbicides [65], for insecticides [66], for antiviral drugs [67] and for antibiotics [68]). The cost of developing new drugs and pesticides has been further increased by the tightening of requirements by regulatory authorities, necessitating a larger number of toxicological, clinical and environmental trials [69]. Hence,
according to Larson [70], it currently takes about 10 years and up to US$1 billion to develop a new antibiotic. Similarly 10 to 12 years are required to develop and launch a new pesticide on the market [71].

At the turn of the 21st century, the combination of approaches such as genomics [72], proteomics [73] and metabolomics [74] with target-based high-throughput screening strategies [71, 75] appeared very promising for the discovery of new drugs and pesticides with little or no impact on environment and health. However, these new methods and strategies have proved relatively unsuccessful, both for antibiotics [76] and for pesticides [77]. The situation is very different for insecticidal toxins, mostly proteins from *Bacillus thuringiensis*, whether formulated for application in sprays or produced by transgenic plants. The number of toxins identified is increasing [78] and the populations of most of the pests targeted remain resistance-free ([66], but see [79]).
Box 2. Strategies for combining molecules over time and space

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

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Antibiotics or antiviral drugs</th>
<th>Insecticides or Bt toxins</th>
<th>Fungicides</th>
<th>Herbicides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Responsive alternation</td>
<td>sequential use</td>
<td>sequence, sequential use</td>
<td>_</td>
<td>sequence, threshold strategy</td>
</tr>
<tr>
<td>Periodic application</td>
<td>cycling, antibiotic rotation, periodic application,</td>
<td>rotation, alternation,</td>
<td>rotation,</td>
<td>rotation</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>n</th>
<th>sequential use</th>
<th>sequential use</th>
<th>sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mosaic</td>
<td>mixing, 50-50 treatment, antibiotic diversity, multiple first-line therapy</td>
<td>mosaic</td>
<td>_</td>
</tr>
<tr>
<td>Combination</td>
<td>combination, antibiotic diversity, simultaneous strategy</td>
<td>mixture, pyramiding</td>
<td>mixture, combination</td>
</tr>
</tbody>
</table>
recommended for several pesticides (e.g. for the control of *Anopheles*, the vector of malaria, [86]).
Box 3. Challenges with Combinations

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

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

A second obstacle for using Combination is that the molecules prescribed by physicians and used by farmers not only control pests and pathogens, they may also injure crops and have undesirable effects on non-target organisms and human health. The World Health Organization (WHO) has reported that there are about three million human cases of pesticide poisoning annually, resulting in 220,000 deaths worldwide [89], hepatotoxicity, neurotoxicity and lipodystrophy [90-92]. Chemical pesticides have a significant impact on non-target plants, fungi and arthropods [93]. Pesticide use can disrupt biological control through direct toxicity [94], indirectly changing the community structure [95] and their predators or parasitoids [96].
A trade-off thus exists between controlling the pest with the right dose and limiting the side effects of treatment. Undesirable effects occur when single molecules are used, but they are probably worsened by the use of combinations, because synergy between molecules [97] can increase the threat to the environment [98] and to human health [99].

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

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

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

<table>
<thead>
<tr>
<th>Strategies</th>
<th>Theoretical studies</th>
<th>Empirical studies</th>
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<tbody>
<tr>
<td></td>
<td>N ¹ 1 &gt; 2 1 = 2 1 &lt; 2 Conditional ²</td>
<td>N ¹ 1 1 = 2 1 &lt; 2</td>
</tr>
<tr>
<td>Combination Responsive alternation</td>
<td>14 11 0 0 3 10 8 2 0</td>
<td>² &gt;</td>
</tr>
<tr>
<td>Combination Periodic application</td>
<td>16 14 0 1 1 8 2 5 1</td>
<td></td>
</tr>
<tr>
<td>Combination Mosaic</td>
<td>7 5 0 1 1 1 1 0 0</td>
<td></td>
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<tr>
<td>Periodic Responsive application alternation</td>
<td>7 3 4 0 0 9 7 2 0</td>
<td></td>
</tr>
<tr>
<td>Periodic Mosaic</td>
<td>11 2 3 5 1 3 2 0 1</td>
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</tr>
</tbody>
</table>

Tableau 1. Comparaisons à côtés des quatre stratégies en termes de leurs capacités relatives à retarder ou prévenir la résistance.
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.