release, different groups might have to be involved in the process on this level, including for example nature conservation organizations, ecologists, entomologists, farmer associations, public health workers, as well as environmental activists or NGOs opposing or not the use of GM or genome-edited organisms.

A central question for the identification of stakeholders will be who pays for the programme and who profits from it. Moreover, are the ones paying also the ones profiting from the deployment? Considering the case of agricultural pest control, who will benefit from the genome-edited insect releases will strongly depend on the production systems, but also on the crop(s) and the pest complexes threatening these crops. It can be assumed that a conventional farmer will mostly profit from the release due to the lower pest burden, resulting in lower costs for insecticide treatments and higher quality harvests with better yields and access to more profitable markets.

In contrast, for the organic farmers, the situation is complex. While they will also profit from the reduced pest insect burden, they might not be allowed to sell their products as they may contain residues of genome-edited material (for example in the form of insect larvae feeding inside the product). In many countries, there is a zero tolerance for GM material in organic food. Depending on how genome-edited material in organic food will be classified and regulated by individual authorities, the use of genome-edited insects might have a negative impact on organic farmers as their product might not be marketable anymore.

Thus, in case of a financing model that involves the producers in the application area, all would pay but not all would benefit. On the other hand, due to the insects' dispersal capacities, neighbouring producers might also profit from the decreased insect burden without paying, for example, if their production areas are located in proximity to a local programme.

Potential sales problems due to food contamination with genome-edited material will not be restricted to local markets but also affect international agricultural trade, where the different tolerance levels for GM/genome-edited material in food will affect the accessibility of markets. The negative impact will therefore not stop at the level of the producers, but it will also affect all downstream links in the trade chain and therefore many more stakeholders (Max-Planck-Gesellschaft 2017). On the one hand, the use of genome-edited insects might close some markets for certain production types (like organic farming), at least for the period of the release. On the other hand, the use of genome-edited insects could also lead to the reopening of markets, if the pest control measure results in the elimination of a quarantine pest, whose presence in the product would have prevented the export or required expensive additional post-harvest measures such as phytosanitary treatments.

Finally, a strong group of stakeholders that needs to be involved is the public, as the insects will be released in their airspace. Involvement of the public in the past has been handled very diversely. The first releases of GM insects were carried out with at best unidirectional public information campaigns but without any possibility to influence the decisions. This caused protest and considerable mistrust against compa-

nies and organizations promoting releases of GMOs (Subbaraman 2011; Baltzegar et al. 2018). Such experiences, step by step, initiated an important reconsideration and change in behaviour towards the public by scientists and organizations. In 2015, the release of GM diamondback moths was halted in New York, USA by the responsible authorities due to public disapproval despite the regulatory approval for the release (Boor 2015; USDA/APHIS 2015; Baltzegar et al. 2018). Similarly, the release of genetically modified mosquitoes by Oxitec Ltd. has been opposed in the target area in the Florida Keys, USA causing Oxitec to finally withdraw the application with the US Environmental Protection Agency (US-EPA) (Klingener 2018).

In Brazil, a major public awareness and engagement campaign was successfully conducted before initiating the release of transgenic mosquitoes in an urban area, emphasizing participatory action and a community-based programme (Capurro et al. 2016). Reconsideration of public involvement goes as far as claims from scientists to make biotechnology research that will affect "everyone" completely transparent from the beginning to build trust (Esvelt 2016, 2017). Certainly, for the success of a programme and its safe, economically worthwhile, and socially and environmentally responsible application, representatives of all relevant stakeholder groups should be identified and involved early in the decision-making process.

8. RECOMMENDATIONS FOR THE SAFE APPLICATION OF GENETICALLY ENGINEERED INSECTS FOR PEST CONTROL

8.1. Evaluation of the Product, not the Technology

From a scientific point of view, the evaluation process of a genome-edited insect should focus on the product, not the technology that was used to create the product. A technology like genome editing can be used to create a variety of genetic modifications, as discussed above, from point mutations and small insertions or deletions, to the introduction of whole transgene constructs. These modifications can also be achieved with other technologies like classical mutagenesis or transgenic approaches, although with different mechanisms. Therefore, the primary evaluation criteria for the product should be the introduced genetic trait, its properties, the projected consequences and benefits, as well as the potential risks in case of field deployment. In a second step, the evaluation should also consider the molecular mechanisms of the technology used, to be able to investigate potential side effects that are characteristic for each technology, including classical mutagenesis and breeding approaches.

A thorough product evaluation in our opinion should include the following points (without claim for completeness):

- Introduced genetic trait and its phenotypic properties – does it fulfil the aspired task?
- Stability of the genetic modification over time, and potential reasons for the trait to fail
- Consequences of a failure of the trait
- Molecular mechanism of the technology and its potential for side effects
- Are off-target mutations present and do the identified changes to the genome have consequences for the trait and its stability, for the genomic stability of the product in the environment upon release, or for the biological quality of the insect?
- Projected benefits of the release of the modified insect
- Potential risks of the deployment of the modified insect into the environment
- Benefits and risks (if any) of current pest control strategies
- Weighting of the benefits and risks of the new strategy against those of the current control strategy and against no intervention
- Possible ecological consequences (e.g. species reduction or elimination).

8.2. Product Evaluation and Risk Assessment

All the above points should be considered in the risk assessment of the product. For the best possible decision concerning a positive impact on society as well as nature, however, a thorough scientific product evaluation and risk assessment will not be enough. It will be crucial to have a wholistic decision-making process that will identify and involve representatives of all possible stakeholders and openly discuss their motivation, expectations and concerns early in the process.

The involvement of as many perspectives and sources of knowledge as possible from the start of the decision-making process will allow a more comprehensive process, that will also have a stronger democratic legitimization (Hartley et al. 2016).

One important point will be the honest, transparent and open discussion of the limits and gaps of the technology and the scientific knowledge and the consequences of a release. This will help to build trust in the scientific process and counteract the hype that can surround new biotechnologies (Caulfield and Condit 2012; Hartley et al. 2016). The 2016 publication by Hartley, based on the discussions of an international and interdisciplinary workshop entitled "Responsible Risk? Achieving Good Governance of Agricultural Biotechnology" held in Norway in November 2015, identifies five points that should be implemented in the scientifically and socially responsible development and application of agricultural biotechnology products: (1) commitment to candour, (2) recognition of underlying values and assumptions, (3) involvement of a broad range of knowledge and actors, (4) consideration of a range of alternatives, and (5) preparedness to respond (Hartley et al. 2016). In the end it should be a common decision of all stakeholders, if the expected positive impact of the product on society and nature is worth taking the risk, even if small.

9. CONCLUSIONS

Genome editing has a high potential for improvement of diverse human life situations. It could be the solution to control many vector-borne diseases, or safely and species-specifically control devastating agricultural pests, to reduce the burden on the human population worldwide. Nevertheless, it has to be studied thoroughly to the point where the technology and its potential drawbacks and side effects are very well understood, current technological roadblocks have been overcome, and the products are evaluated carefully according to well-defined regulations.

Regulations for genome-edited insects and other genome-edited products should be in the best interest of society based on scientific data acquired, rather than being based on an opinion from groups that are not independent. Therefore, also the underlying interests of institutions funding genome editing research should be critically analysed before they are allowed to give "public" views at any stage in the decision-making process. False information policies lead to the stalling of academic as well as industrial research, and in the case of Germany killing important innovation and economic development that will instead move to more open and innovation-friendly countries.

Genome editing is still in its infancy, and the consequences of tampering with gene function are not yet well understood, even when editing genes that researchers thought to be well studied. The gene networks are far more complex than so far assumed and meddling with them at this stage can have unforeseen consequences. While this lack of full understanding urges progressing with caution, it poses at the same time a strong demand for more research to understand the mechanisms involved, learn about side effects, and re-engineer the technology accordingly to make genome editing the safest possible technology. What would be counterproductive is a substantial restriction or even shutdown of the research due to uninformed or even misinformed fears and false information, which prevents better understanding and improvement of gene editing technologies.

In no way this should be a charter to do anything that is possible, but to base decision-making of every new technology on transparent science, facts, and comparing them to existing technologies to allow decisions like "are they an improvement or not?" This train of thought has been used in plant breeding for a long time, where only seeds with proven improvements over the existing ones are allowed on the market.

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SYNTHETIC SEX RATIO DISTORTERS BASED ON CRISPR FOR THE CONTROL OF HARMFUL INSECT POPULATIONS

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SUMMARY

Since the overall reproductive output of a population is typically determined by the fertility of its females, which are rate-limiting in gamete production, a successful way to genetically control a population should involve artificially biasing the sex ratio towards males. In male heterogametic species, this could be achieved by the expression of a transgene-encoded endonuclease during spermatogenesis that would target and "shred" the X chromosome at several loci. This would prevent the transmission of X chromosome bearing gametes to the progeny, generating only males. Recent developments in molecular and synthetic biology have provided genome editing tools with great potential to engineer the genome of different species. Given the targeting flexibility of CRISPR-based endonucleases, it may now be possible to test whether X chromosome shredding has the potential to become a universal strategy to genetically control a wide variety of insect pests, of both agricultural and public health relevance.

Key Words: Meiotic drive, sex ratio distorters, biased sex ratio, gene editing, genome editing, X-shredding, genetic control, spermatogenesis, male-determining genes, gene drive, selfish chromosomes

1. INTRODUCTION

In most sexually reproducing organisms, males and females are generated in approximately the same numbers. Fisher's principle states that the sex ratio is in equilibrium when an individual spends the same amount of energy to produce equal numbers of males and females. When the ratio is different from the equilibrium, the less predominant sex, or rather genes determining development towards this sex, will have an advantage that will last until the equilibrium is re-established (Fisher 1958). As the balanced sex ratio is approached, the advantage associated with producing the

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rarer sex wanes, and the equilibrium is re-established. Consequently, novel genetic traits that bias the sex ratio towards one sex gain a short-term advantage but are eventually counterbalanced by a neutralizing evolutionary force in the form of drive suppressors that evolve on the autosomes or on the Y chromosome. Sex ratios can therefore be portrayed as the dynamic outcome of an ongoing evolutionary "tug of war" (Argasinski 2013). Fisher's principle only applies in those cases where the sex-ratio is controlled by genes acting in the homogametic sex, or by autosomal genes acting in the heterogametic sex, in XX-XY and ZW-ZZ systems.

In a population of a sexually reproducing organism, a dramatic sex bias towards one of the two sexes usually decreases the population's overall fertility. Since the overall reproductive output of a population is typically determined by the fertility of its females, which are rate-limiting in gamete production, a successful way to genetically control the population could involve artificially biasing the sex ratio towards males (Hamilton 1967). Such a genetic control strategy could reduce the size of harmful animal populations, such as agricultural insect pests or disease-vector species, or even result in the suppression or collapse of the population before suppressor alleles can arise to re-establish a balanced sex ratio. In control programmes, a male-biased sex ratio would also be favourable because females are often responsible for the damage (e.g. they are often the vectors of human parasites or viruses (e.g. mosquitoes) or lay eggs in agricultural products (e.g. fruit flies).

Hamilton was among the first to suggest how genetic sex ratio distorters (SRDs) could be applied to eradicate mosquito populations, and that under certain conditions, non-Fisherian sex ratios could arise and yet be maintained. He considered a heterogametic species with males (XY) harbouring a mutant Y chromosome that can bias fertilization in its favour at the cost of the X chromosome. Males carrying such a Y mutation would only produce sons. As a result, the mutant Y chromosome would gain a selective advantage and spread within the population, rendering it increasingly male-biased at each generation. The decline in female numbers would result in a decrease in size and eventually the collapse of the population (Hamilton 1967). In this example of SRD, an invasive Y chromosome interferes with the production of X-bearing gametes during spermatogenesis and spreads through the population in a self-sustaining manner. Hamilton's thought experiment was inspired by observations of natural populations of mosquitoes, where Craig et al. (1960) reported that a SRD transmitted by males was responsible for a male bias in *Aedes aegypti* (L.), the yellow fever mosquito. Observations in other species since then have also identified an X chromosome bias and thus more female progeny in some populations of *Drosophila simulans* Sturtevant (Mercot et al. 1995). Importantly, it is often difficult to identify the existence of a SRD in a population because local suppressors evolved to counteract it – had they not the population would have likely disappeared.

For the purpose of this chapter, it is important to distinguish such driving (invasive) SRDs from traits that bias the sex ratio, but that are inherited in a non-driving standard Mendelian fashion (Fig. 1). Both types of SRDs, in turn, are distinct from female killing systems (FK) that also result in a biased sex ratio in progeny, but they do so at the expense of reproductive output because of post-zygotically lethality of female offspring.

Unlike the bias generated by female killing systems (FK), SRDs operate pre-zygotically during gametogenesis and thus do not result in an overall reduction of male fertility. The main advantage of driving SRDs, as far as genetic population control is concerned, is their invasiveness. In the absence of resistance against the drive, the driving Y chromosome will eliminate the X chromosome in sperm and eventually lead to population collapse due to the lack of females.

Nevertheless, non-invasive SRD traits also have significant potential for genetic control, although they are eventually lost in the absence of continuous releases. They are advantageous in particular when compared to other forms of inundative genetic control approaches such as the Sterile Insect Technique (SIT) (Dyck et al. 2021) or its transgenic cousin, the Release of Insects carrying a Dominant Lethal (RIDL technology) (Thomas et al. 2000) as they require smaller effective releases (Schliekelman et al. 2005).

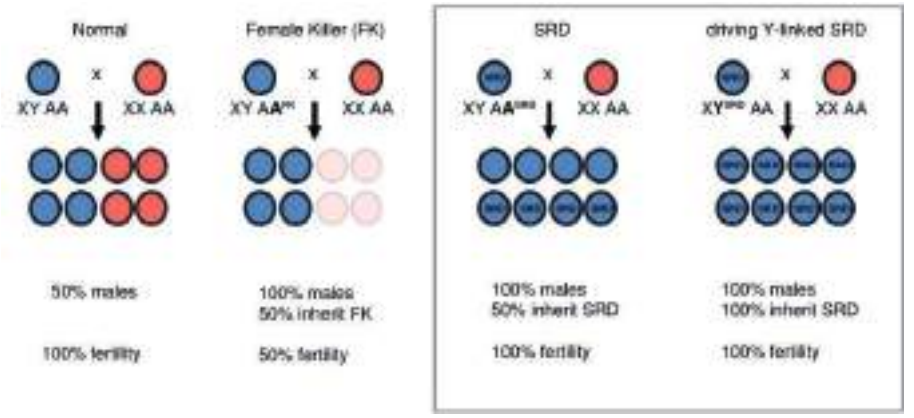

Figure 1. Male-biased sex ratio distorters (SRDs), unlike female killing systems (FK), bias the reproductive sex ratio towards males, while ideally maintaining full fertility.

SRD is a form of segregation distortion (SD) or meiotic drive, a term that also encompasses transmission of anomalies that are not strictly meiotic but that alter the normal process of meiosis, generating a gametic pool with one type of allele in excess (Zimmering et al. 1970). When SDs are physically linked to sex-determining loci or sex chromosomes, meiotic drive will result in an unbalanced sex ratio in the next generation. Reduced recombination between sex chromosomes favours the emergence of meiotic drive systems along them and indeed sex chromosome SDs are abundant in nature (Hammer 1991; Lyttle 1991).

SRDs occur mostly in association with male heterogamy, and usually, it is the X chromosome that drives against the Y chromosome, as a consequence of which males produce a strongly female-biased progeny. However, SRD systems in which the X drives against the Y chromosome, as in *Drosophila*, are not efficient in insect control programmes. Not only does population collapse proceed at a slower pace and is delayed by male polygamy, but it could result in short-term spikes in population size due to the female-biased progeny (Novitski 1947; Hamilton 1967).

Y-linked SRDs have been found to occur in nature in the culicine mosquitoes *Ae. aegypti* L. and *Culex pipiens* L., both of which harbour homomorphic sex chromosomes (Gilchrist and Haldane 1974; Fontaine et al. 2017). Males of these two species are heterozygous at the sex-determining locus (Mm) which is located in chromosome 1. The gene determining male sex was recently described in *Ae. aegypti* (Hall et al. 2015; Turner et al. 2018). The meiotic drive locus, M^D, is closely associated to M, acts in *trans,* distorting expression at a responder locus that is proximal to and indistinguishable from m, the locus that is homozygous in females. Subtle enhancers and suppressors of M^D strength have been discovered on all autosomes of *Ae. aegypti,* as have responder loci of varying sensitivity (Wood and Ouda 1987; Wood and Newton 1991; Cha et al. 2006).

Specific crosses involving the field-caught T37 strain of *Ae. aegypti*, result in a male-biased population of about 85% (Shin et al. 2012). Initial cage-population experiments to assess the suitability of the M^D locus for controlling natural populations of *Ae. aegypti,* showed females with developed resistance to M^D. Although the mechanism responsible for the resistance remains unknown, the level of distortion ultimately attained was insufficient to achieve effective population control (Hickey and Craig 1966; Robinson 1983).

Since natural resistance to M^D is common in the field, only native populations that are highly sensitive could be targeted. The efforts involved in the many attempts to apply M^D for insect control have highlighted the problems that could arise by using naturally occurring distorters for which resistance or rather counteracting alleles are already in existence. Also, their potential to be transferred to other target species is unclear as SRD and responder loci are expected to have co-evolved. Thus, efforts have intensified to develop entirely synthetic SRD strategies, which will be the focus for the remainder of this chapter.

2. SYNTHETIC SEX RATIO DISTORTERS BASED ON THE X CHROMOSOME SHREDDING MODEL

Synthetic distorters have the advantage of being unaffected by some, or all of the suppressor alleles that may exist to counteract naturally circulating distorter alleles. This is the case of synthetic SRD systems designed to circumvent the established sex determination pathway, operating independently of it. In the naturally occurring SRDs in *Cx. pipiens* and *Ae. aegypti* mosquitoes, cytological observations revealed that during the early stages of male meiosis, the X-equivalent chromosome harbouring the m locus are fragmented. This is accompanied by an increase in M-bearing gametes and a reduction in the number of females born in the next generation (Newton et al. 1976; Sweeny and Barr 1978).

The observations of X chromosome fragmentation suggested that a similar system for SRD could be artificially created through endonuclease-mediated cleavage of the X chromosome during male meiosis. In male heterogametic species, this could be achieved by the expression of a transgene-encoded endonuclease during spermatogenesis that would target and cut the X chromosome at several loci (Fig. 2). Consequently, only gametes with the Y chromosome would be produced or would be functional to achieve fertilization and only males would be generated (Burt 2003).

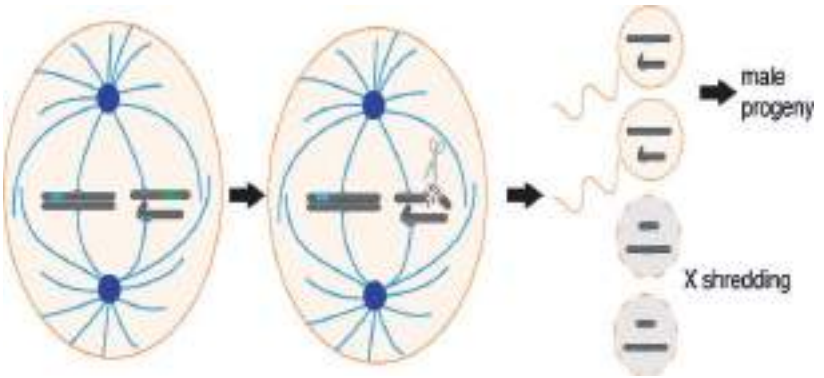

Figure 2. A multicopy target sequence (green bars) on the X chromosome is targeted by an endonuclease (scissors) during spermatogenesis. Shredding of the X chromosome favours the unaffected Y-bearing sperm and results in a male-biased progeny. Blue and red boxes are endonuclease and gRNA genes, respectively.

2.1. I-PpoI as the First Synthetic Sex Ratio Distorter in the Malaria Mosquito

A transgenic SRD trait was first developed and tested in the malaria mosquito *Anopheles gambiae* Giles, expressed from autosomal locations (Windbichler et al. 2007, 2008; Klein et al. 2012). It utilized the *Physarum polycephalum* Schwein I-PpoI endonuclease, which was driven by the *β2-tubulin* promoter that is specific to spermatogenesis. I-PpoI is an intron-encoded endonuclease mapping in the 28S rDNA locus that selectively cleaves ribosomal DNA sequences.

In *An. gambiae*, the ribosomal repeats are localised exclusively on the X chromosome making the targeting and cutting highly specific to the X chromosome. The expression of wild-type I-PpoI during spermatogenesis resulted in the cleavage of the X chromosome, but also in male sterility. It was found that during fertilisation, mature sperm carrying stable I-PpoI transfers endonuclease protein to the egg, determining the shredding of the maternal X chromosome and thus sterility.

To reduce the *in-vivo* half-life of I-PpoI and limit its activity to male meiosis, Galizi and colleagues (Galizi et al. 2014) generated a series of protein variants by site-directed mutagenesis. They modified amino acid residues involved in the zinc-binding core formation, protein packaging and protein dimerization. Next, they generated transgenic mosquitoes carrying autosomal insertions of each variant of the I-PpoI protein and analysed hatch rate and percentage of SRD in the progeny of hemizygous males crossed to wild-type females. W124A strains, with a mutation in the dimerization domain of I-PpoI, showed the highest hatch rate and SRD in the progeny corresponding to 97.4% males and a hatch rate close to the control. The SRD phenotype was stably inherited for four consecutive generations by transgenic sons. In five independent laboratory cage experiments with such males it was at least two orders of magnitude more efficient than sterile males. The cage studies also showed that releases of hemizygous distorter males at an overflooding ratio of 3x to wild-type males, was sufficient to eliminate the *An. gambiae* cage populations within six generations.

2.2. CRISPR-Cas9-Based X Chromosome Shredding to Induce Sex Ratio Distortion

While the I-PpoI system is a working model for an X chromosome shredder in *An. gambiae*, it is not transferable to other organisms unless they share the same location of the target rDNA sequences on the X chromosome.

Recent developments in molecular and synthetic biology have provided genome editing tools with great potential to engineer the genome of different species. The most promising is the RNA-guided CRISPR-Cas9 (Clustered Regularly Interspersed Short Palindromic Repeats-CRISPR-associated 9) endonuclease system (Mali et al. 2013). Here, a guide RNA (gRNA) "guides" the endonuclease to the complementary DNA sequence, which is digested by the enzyme generating double-strand breaks. Cas9 can be used to cleave any complementary target DNA harbouring a PAM (Protospacer Adjacent Motif) sequence, a motif consisting of the three-base-pair, NGG (any nucleobase followed by two guanine nucleobases).

In addition, other RNA-guided endonucleases have recently been discovered, such as Cpf1, smaller than Cas9 and A/T rich genomic-region specific, that may also prove to be as versatile as the Cas9 system (Zetsche et al. 2015). Compared to systems such as zinc-finger nuclease (ZFN), TALE nucleases (TALEN) and homing endonuclease genes (HEGs), RNA-guided systems do not require alteration of the protein to recognize the target sequences. Instead, gRNAs consisting of 18-20 RNA nucleotides are sufficient to lead the endonuclease to its target. Thus, endonucleases with novel specificities can be generated more easily and a larger number of genomic loci can be targeted and cleaved in parallel. Lastly, different gRNAs can be used to target multiple sites simultaneously, thus decreasing the odds of developing resistance alleles.

The CRISPR-Cas9 system, because of its specificity and flexibility, has been tailored to modify the genomes of different organisms, including yeast (DiCarlo et al. 2015), plants (Li et al. 2013), worms (Frokjaer-Jensen 2013), fruit flies (Gratz et al. 2013; Gantz and Bier 2015), the jewel wasp *Nasonia vitripennis* Walker (Li et al. 2017), mosquitoes (Basu et al. 2015; Gantz et al. 2015; Kistler et al. 2015; Hammond et al. 2016), zebrafish (Hwang et al. 2013), mice (Shen et al. 2013), monkeys (Niu et al. 2014), and human cells (Cong et al. 2013).

Recently, CRISPR-Cas9 was successfully used to act as a SRD system in *An. gambiae*. As a follow-up of the I-PpoI work, Galizi and colleagues, designed a transformation construct containing the *cas9* gene under the control of the *β-tubulin* gene promoter, and a gRNA targeting an X chromosome-linked rDNA sequence distinct from the original I-PpoI target (Galizi et al. 2016). This site was selected because it was conserved among closely related species of the *An. gambiae* complex, but crucially, was absent from more distantly related insects such as *Drosophila*. Similarly, to the observations with I-PpoI, the progeny of males from all tested transgenic lines displayed a high SRD with values ranging from 86.1% to 94.8% males. The fertility of transgenic males of almost all lines was similar to the wild-type, with hatching rates between 83.6% and 93.2%. Furthermore, although the *β-tubulin* promoter was used to regulate the expression of both Cas9 and I-PpoI (discussed above) endonucleases, the Cas9 protein was not paternally carried over into the fertilised embryo, thus increasing the hatching values and simplifying the generation of SRD.

Similar to I-PpoI, five consecutive generations of transgenic males stably inherited the SRD phenotype. As was observed for I-PpoI, however, the paternal X chromosome of rare survivor females exhibited both target sequence repeats that were intact indicating that not all of the targets were cleaved, as well as target sequence repeats that were found to be resistant to *in-vitro* re-cleavage as a possible consequence of DNA miss-repair. This is consistent with CRISPR-Cas9-mediated generation of resistant alleles by non-homologous end joining (NHEJ) or microhomology mediated end joining (MMEJ), repair mechanisms in DNA double-strand breaks that have been shown to adversely affect CRISPR-based gene drive systems (Hammond et al. 2016). For CRISPR-based SRDs, the likelihood of miss-repair leading to overall resistance to X chromosome shredding and consequently to the failure of population control is greatly reduced as a higher number of X chromosomal sites are targeted simultaneously.

2.3. Potential for Establishing CRISPR-Cas9-Based Sex Ratio Distorters in Other Species

Given the flexibility allowed by CRISPR-based endonucleases, it may now be possible to test whether X chromosome shredding has the potential to become a universal strategy to genetically control a wide variety of heterogametic insect pests, of both agricultural and public health relevance. However, the proof-of-principle in *An. gambiae* relies on the peculiar localisation of the mosquito's rDNA genes, which are confined to the X chromosome, and the availability of an endonuclease that targets these repeats, two characteristics unlikely to be shared by many other insect species.

For X chromosome shredding to become a more widely used approach, what is required is a method to identify motifs repeated exclusively on the X chromosome, and the ability to engineer endonucleases able to target and cleave these sequences in the male germline.

Recently, a bioinformatic pipeline called Redkmer (for Repeat Extraction and Detection based on kmers, all the possible sub-sequences of length k from a read obtained through sequencing) used for DNA sequence analysis and comparison, was developed for X chromosome target sequences shredding by CRISPR endonucleases (Papathanos and Windbichler 2018). It relies on long and short read sequencing technology and can identify highly abundant X-specific sequences. Because genome assemblies typically exclude highly repetitive sequences such as satellite DNA, it was designed to not rely on high-quality assembled genomes. Indeed, for many target insect species, high-quality genome assemblies are not available.

The Redkmer pipeline requires as input only whole genome sequencing (WGS) data based on long (e.g. PacBio) and short (e.g. Illumina) unassembled reads of the relevant insect. WGS for the long reads is performed only on male individuals, while short read data are generated from both, male and female individuals, independently. For the selection of highly abundant X chromosome kmers, the pipeline relies on two features: (1) WGS elements mapping on the X chromosome that occur on average twice as often in female compared to male data; (2) overall sequence abundance with X chromosome specificity.

Preliminary data in *An. gambiae* confirmed the potential of the bioinformatic pipeline, revealing X-specific and abundant kmers that overlapped with the known rDNA cluster. The now affordable costs of next-generation sequencing and the increase in computer power, combined with the flexibility of the CRISPR-Cas9 system, will ease the application of SRD to genetically control disease vectors and economically or ecologically damaging invasive species.

3. CONSIDERATIONS ON THE USE OF SYNTHETIC SEX RATIO DISTORTERS

SRD systems are subject to two essential limitations. First, SRDs will only work in sexually reproducing species. Second, like the SIT, maintaining SRD traits within a target population requires scheduled releases of new transgenic individuals that need to be continuously reared in considerable numbers rendering the approach costly. As mentioned, this could be eased by linking the SRD trait to the male Y chromosome. Thus, in this case, all male offspring will inherit the transgene that will spread invasively.

For scaling up and potential field testing of the non-driving SRD traits described above, transgene expression should be conditionally repressed. This would enable mass-rearing, as constant backcrossing of a constitutively expressed SRD trait at each generation is unlikely to be a practical method. Conditional expression systems have been successfully used in the field, in combination with dominant lethality, but should be adapted to work in conjunction with CRISPR-based constructs.

3.1. Difficulty in Turning Non-driving SRD into Driving-SRD Traits

All synthetic SRD systems described so far are non-driving autosomal distorters. Moving autosomal distorters to the Y chromosome is, however, far from the straightforward proposition it appeared to be initially. All active distorter transgenes examined by Galizi et al. (unpublished) had the construct inserted on an autosome. Lines with the transgene integrated on either the X or Y chromosome did not show a significant level of I-PpoI expression in the testes. This was explained by the repressive effect of meiotic sex chromosome inactivation (MSCI) that silences unpaired chromatin during meiotic stages of spermatogenesis. Inactivation of transgenes integrated on the X chromosome has been described before in mosquitoes (Magnusson et al. 2012; Papa et al. 2017). MSCI may turn out to be a significant obstacle in turning non-driving SRD into driving-SRD traits, as its biological function may be that of policing SD traits.

3.2. Advantages of the Standard Autosomal SRD Systems

One advantage of the standard autosomal SRD systems based on RNA-guided endonucleases is that they can be engineered to work, at least in principle, in most heterogametic species and therefore suppress the population of many target organisms.

Recently the precise deletion of chromosomes in cell lines of mammals (Adikusuma et al. 2017) has been demonstrated and indicates that this system could also work in higher organisms. From an ecological standpoint, a second advantage is the reduced risk of invasion compared to driving Y-linked SRDs or compared to other gene drive elements that are being developed for the purpose of population replacement (Gantz and Bier 2016; Hammond et al. 2016). Once released, the frequency of the SD alleles decreases instead of increasing in the population, a desirable feature to avoid permanent ecosystems alterations (Oye et al. 2014).

3.3. Engineering Multiple gRNAs to Reduce Generation of Resistance Alleles

In RNA-guided SRD systems there is the need to understand the likelihood at which resistant alleles will develop in the target sequences (Bull 2015). The tendency of natural selection to favour equal sex ratios exerts pressure on the NHEJ (non-homologous end joining) repair system that may generate resistance alleles via insertion and deletions in target sequences. In addition, natural sequence polymorphism between individuals of the same population could also prevent cutting. Therefore, to reduce the growth of resistance alleles, it is critical to engineer different gRNAs cutting simultaneously, including some that target conserved regions of essential genes. The degree to which X chromosome shredding systems can rely on the existence of numerous repetitive sequences of the same target on the X chromosome, may directly determine the likelihood of the rise of resistance alleles, although further research is required in this area.

3.4. Environmental, Ecological, and Regulatory Challenges

The release of transgenically modified organisms for population control is challenged by a series of environmental, ecological, and regulatory difficulties. Transgenic males should be able to mate and scout for females to the same extent as wild-type males (Lacroix et al. 2012). A different behaviour would drop the chances of spreading to remote regions reducing the success of population control. Different factors can decrease the competitiveness of males such as mass-rearing, inbreeding, transgene expression and its insertion site in the genome (Catteruccia et al. 2003; Reed et al. 2003; Baeshen et al. 2014). The use of the PhiC31 integration system that provides precisely mapped docking sites, has helped to reduce the position effect on gene expression (Amenya et al. 2010).

Genetic variation of wild-type individuals is another feature that can influence the success of the release operation. Polymorphisms in the gRNA target site can compromise the ability of the endonuclease to cleave the DNA. Different strains can have slightly dissimilar mating behaviours or live in distinctive ecological niches making their control even more difficult. These are all features that should be considered when applying genetic engineering to control vector-borne disease or agricultural pests.

Ultimately, the release of transgenically modified individuals causes environmental and safety challenges that should be addressed in each individual case. Ecological and molecular containment strategies should be considered when designing RNA-guided SRD systems. The chances of the SRD trait spreading to non-targeted species, and horizontal transfer of the transgenes must be safely reduced before releasing the modified individuals (David et al. 2013; Nielsen, this volume). Species-specific targeting sequences and promoters to regulate the endonuclease expression should also prevent lateral gene transfer beyond target populations (Oye et al. 2014).

Confined laboratories, with high containment levels and only small-field tests, should initially be used to determine the safety and specificity of the transgene modification. An open discussion with regulatory agencies, the scientific community and the public is fundamental to inform on the risks and benefits of using genetic-engineering technologies to control vector-borne diseases and alleviate the economic burden inflicted by agricultural pests (Oye et al. 2014).

4. OTHER APPROACHES TO ACHIEVE SYNTHETIC SEX RATIO DISTORTION

We have focussed our discussion on X chromosome shredding as a paradigm for developing synthetic SRDs. However, other approaches are also conceivable to achieve the same goal. Recently, the long-elusive male-determining genes of a number of important pest and vector species have been identified. These include the Y chromosome-linked male-determining genes in mosquitoes *An. gambiae* and *An. stephensi* Liston, the M-locus linked gene in *Ae. aegypti*, the mobile splicing factor in *M. domestica* L. and the MoY factor in *Ceratitis capitata* (Wiedemann) (Hall et al. 2015; Criscione et al. 2016; Krzywinska et al. 2016; Sharma et al. 2017; Meccariello et al. 2019).

Knowing the sex-determining genes in each of these species will allow designing synthetic SRDs. In addition, the use of a nuclease-based gene drive could ensure the transmission of the synthetic SRDs to the entire progeny and bring more rapidly the population to collapse (Kyrou et al. 2018; Häcker and Schetelig, this volume).

Finally, caution should be used when sex-determining genes play functional roles in other essential pathways, such as in dosage compensation. Their multiple functions may interfere with some strategies to manipulate sex ratios, for example through the lethality of transgenic male-determination signals on chromosomes other than the Y chromosome.

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THE USE OF SPECIES DISTRIBUTION MODELLING AND LANDSCAPE GENETICS FOR TSETSE CONTROL

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SUMMARY

Trypanosomosis is one of the major constraints to rural development in sub-Saharan Africa. Studies of the species distribution and landscape genetics allows for targeting tsetse-infested areas and optimizing the management of tsetse fly populations. In this chapter, a review is provided on 1) available spatial tools and landscape genetics, 2) the use of tsetse distribution models to rank populations according to their level of isolation to assist identifying populations that can be targeted for eradication, and 3) the use of tsetse distribution models to optimize tsetse control efforts within an area-wide integrated pest management context.

Key Words: Glossina, habitat suitability, African trypanosomosis, nagana, sleeping sickness, sub-Saharan Africa, area-wide integrated pest management, spatial modelling, Maxent, distribution models, friction models, population genetics

1. INTRODUCTION – THE TSETSE AND TRYPANOSOMOSIS PROBLEM

Tsetse flies (*Glossina* spp.) are widely distributed in sub-Saharan Africa and inhabit semi-arid, sub-humid and humid lowlands in 37 countries across the continent with a potential distribution range of some 8.7 million km^2 (Rogers and Robinson 2004). They transmit trypanosomes, the causative agents of sleeping sickness (human African trypanosomosis, HAT) and nagana (African animal trypanosomosis, AAT).

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Together, the animal and human diseases pose health threats and a great economic burden to vast regions of sub-Saharan Africa where they are endemic (Swallow 1999; Diall et al. 2017).

The number of HAT cases has substantially declined in the last 15 years, mainly through increased disease surveillance and treatment of affected patients (WHO 2013), and since 2012 in *Trypanosoma brucei gambiense* HAT foci, thanks to increased vector control (Courtin et al. 2015; Mahamat et al. 2017). Whereas in 2000 there were more than 25 000 newly reported cases, this had decreased to 7106 new cases by 2012. In 2018, less than 1000 new cases of HAT were reported to the World Health Organization (WHO 2019), which represents the lowest number of sleeping sickness cases ever recorded. In some countries with ongoing conflicts, there is obviously the likelihood of under-reporting of cases. There is, however, general consensus that the complete elimination of HAT as a public health problem can only be possible through the inclusion of an effective vector management component (Solano et al. 2013; Feldmann et al. 2021).

Contrary to the advances made with the management of HAT, the AAT continues to represent the greatest animal health constraint to improved livestock production in sub-Saharan Africa, causing economic losses amounting to USD 4.75 billion annually and putting approximately 50 million cattle at risk (i.e. milk and meat production) (Swallow 1999; Scoones 2016). The disease also prevents the integration of crop farming and livestock keeping, a crucial component for the development of sustainable agricultural systems (Alsan 2015).

Early death can result in chronically infected animals if AAT is not treated, and at least three million cattle and other domestic animals succumb to the disease each year (Hursey and Slingenbergh 1995). When not lethal, AAT brings livestock into a chronically debilitating condition and reduces fertility, weight gain, meat and milk production by at least 50%, as well as the work efficiency of oxen used to cultivate the land (Budd 1999; Swallow 1999; Shaw 2004). The disease has also indirect negative effects on the development of commercial domestic and livestock production, i.e. it discourages the use of more-productive exotic and cross-bred cattle, depresses the growth and distribution of livestock populations, reduces the potential opportunities for integration of livestock keeping and crop production through less draught power to cultivate land and to transport farm products to market, and less manure to fertilize (in an environment-friendly way) soils for better crop production.

In addition, the scarcity of domestic animals leads to a serious shortage of animal protein for human consumption and as people tend to avoid areas infested with tsetse flies, they affect human settlement (Shaw 2004). Consequently, tsetse flies and the AAT it transmits are considered as one of the root causes of hunger and poverty in about one third of the African continent (Feldmann et al. 2021). AAT therefore is an important limiting factor to reach Sustainable Development Goals 1 (No Poverty) and 2 (Zero Hunger) (UN 2019).

Understanding the distribution of tsetse flies in space and time is essential in selecting the most appropriate intervention strategies for the area-wide management of tsetse populations in different parts of sub-Saharan Africa. Despite substantial efforts for more than a century, deliberate efforts to reduce the vast tsetse belt have had very limited success (Vreysen et al. 2013).

In past decades, spraying of insecticides was effective in controlling tsetse fly populations in certain areas of the African continent, e.g. to stop the advancing front of tsetse flies in south-eastern Zimbabwe and south-western Mozambique between 1962 and 1974 (Robertson and Kluge 1968; Lovemore 1972, 1973, 1974; Robertson et al. 1972), and to eliminate tsetse flies in Zimbabwe (Jordan 1986), Nigeria (MacLennan and Kirby 1958), Botswana (Davies 1980), Zambia (Paynter and Brady 1992), the Zambezi region (formerly Caprivi Strip) of Namibia, and in Malawi (Vale 1999). However, although the spraying of non-residual pyrethroids as ultra-low volume formulations either using ground fogging techniques or the sequential aerosol technique (SAT) was until recently still used in Burkina Faso, Ethiopia, Ghana, Guinea and Zimbabwe (Bouyer and Vreysen 2018), the spraying of residual insecticides is no longer recommended anymore on environmental grounds.

Whereas in the past, suppression methods were often used alone and against only certain segments of the tsetse population, in the last decades it has become evident that more sustainable tsetse population management can be obtained when applying two compatible strategies, i.e. area-wide integrated pest management (AW-IPM) (Vreysen et al. 2007) and the phased conditional approach (PCA) (Bouyer and Vreysen 2018). The management of tsetse fly populations can be implemented using two basic approaches, i.e. on a localised field-by-field basis or on an area-wide basis (total population management) (AW-IPM) (Hendrichs et al. 2007; Klassen and Vreysen 2021). AW-IPM is an approach that consists of a coordinated effort against all sub-units of a target pest population in an ecosystem before the pest population has reached damaging proportions. Local field-by-field pest control is a reactive effort when the pest population reaches damaging levels and is carried out individually and independent of the action of neighbouring farmers. These two strategies have different objectives. Whereas population suppression (the reduction of the insect pest density below a threshold preventing damage or disease transmission) can be the objective in both cases, local population elimination (eradication would signify the elimination of all populations of a given insect species from the planet) is only possible using the second approach, with higher costs but proportional longer-term impacts (Vreysen 2006; Bouyer et al. 2013).

The AW-IPM approach minimizes the risk of reinvasion, as areas that are of no interest to the farmers are also targeted. This approach usually requires several years of planning and a specialised organization with dedicated staff to implement the control activities, in an adaptive management scheme. The AW-IPM can benefit from advanced technologies such as geographic information systems (GIS), population genetics (increasingly being used for designing and implementing tsetse control efforts; Bouyer et al. 2021), remote sensing and aerial dissemination techniques (Vreysen 2006; Dicko et al. 2014; Klassen and Vreysen 2021).

This chapter highlights the integration of species distribution modelling and landscape genetics to facilitate the management of tsetse populations. First, it presents a general overview of spatial tools and landscape genetics. Second, it presents how modelling the distribution of tsetse populations and ranking them according to their level of isolation can help to identify populations that can be targeted for eradication. Finally, it presents how tsetse distribution models can be used to optimize tsetse control efforts within an AW-IPM context.

2. GENERAL OVERVIEW OF SPATIAL TOOLS AND LANDSCAPE GENETICS

2.1. Spatial Modelling and Geographic Information Systems

Spatial tools have long been important to natural resource applications. The GIS, global positioning system (GPS), and remote sensing (RS) are spatial tools that have become more and more important for decision-making in the control of diseases, i.e. to locate important target sites, to predict population change based on climatic trends, to report potential anomalies, but also to analyse landscape patterns, disaster management, etc. Data can, in many instances, only be fully understood when they can be placed in a geographic context. Hence the benefits that can be derived from using GIS, which are computer-based tools that analyse, store, manipulate and visualize geographic information, usually in a map (Bouyer et al. 2021). GPS is a satellite navigation system used to determine the exact position of an object, whereas RS aims at providing access to a range of satellite-derived data products about the earth's surface using electromagnetic sensors.

These geospatial tools have made the design and the implementation of AW-IPM programmes and disease control much more effective and cost-efficient. The importance of geomatic tools to assist various stages of planning and application of the Sterile Insect Technique (SIT) as part of an AW-IPM approach is presented elsewhere (Bouyer et al. 2021) and includes the selection of project sites, planning of pre-intervention surveys and feasibility assessments before the start of the operational implementation campaign. Geomatic tools are also essential to monitor and analyse insect populations during area-wide control efforts to be able to implement adaptive management (Vreysen et al. 2013).

2.2. Landscape Genetics Approach

Landscape genetics, that associates tsetse population genetics with spatial tools, is an innovative and emerging approach that enables understanding how geographic and environmental features structure genetic variation at the population and individual scales (Feldmann and Ready 2014). Genetic markers with varying temporal or spatial resolution can be used to implement landscape genetics, depending on ecological questions (De Meeûs et al. 2007). This approach can not only be used for improving ecological knowledge, but also for explaining observed spatial genetic patterns as clines, isolation by distance, genetic boundaries to gene flow, metapopulations and random patterns (Manel et al. 2003), in order to manage properly the genetic diversity.

Various approaches have been used to quantify the spatial structure relying on landscape ecology. However, most involve the incorporation of the notion of landscape resistance or friction (i.e. the impediments to gene flow) caused by landscape features. The most common approach employed involves measuring the "cost" distance between populations sampled based on one or more alternative landscape resistance models (Manel et al. 2003).

Informing on the resistance of landscape to movement is essential to refine species distribution models. The geographic distribution of a given species can be seen as the

intersection between biotic (B), abiotic (A) and movement (M) factors in a BAM diagram (Soberón and Peterson 2005). However, most species distribution models neglect the latter (Barve et al. 2011), often because they are based on presence/absence data and rarely include genetic data. It is however essential to account for movement, as a given landscape can be suitable for a given species, but not inhabited because it is out of reach for this species. Moreover, friction should not be mapped based on expert knowledge, as this is very subjective and therefore essentially unpredictable.

Tsetse studies have demonstrated that the environmental parameters driving landscape suitability are totally different from those driving landscape friction (Bouyer et al. 2015). This can be described as the "salamander paradox", i.e. if a forest salamander finds itself at 100 m from the edge of a forest, in an area where the ground is bare and very inhospitable to its survival, it will either die quickly or move as fast as possible to the forest. Conversely, if the salamander finds itself in the forest, it will be in a location with all suitable conditions and it will not disperse much. Therefore, the friction of the forest will be higher than that of the bare ground, which seems counter-intuitive (Peterman et al. 2014).

Insect pest populations can be structured at micro-geographic scales, which must be accounted for to optimize control. For example, the inclusion of population genetics data in control programmes against tsetse populations of the *palpalis* group in West Africa provided information on the level of genetic isolation of the target populations from the neighbouring ones, which allowed informed decisions for developing control strategies. However, for population genetic tools to provide accurate inferences, individuals must be sampled at the smallest scale possible and the molecular markers carefully selected (Solano et al. 2010a).

3. APPLICATION OF DISTRIBUTION MODELS AND LANDSCAPE GENETICS FOR TSETSE CONTROL

3.1. Mapping Landscape to Identify Isolated Tsetse Populations

Understanding how geographic and environmental features structure genetic variation of tsetse populations is essential for the development of intervention strategies of these cyclical vectors of HAT and AAT in sub-Saharan Africa. Evidence of restricted or absence of gene flow allows genetically isolated islands to be identified (Solano et al. 2009), or isolated ecological population islands (Solano et al. 2010b), from where the tsetse populations present could be eradicated without risk of reinvasion.

For example, two environment-friendly tsetse eradication campaigns achieved the creation of a sustainable tsetse-free zone, i.e. (1) on the Island of Unguja, Zanzibar where an AW-IPM strategy was used to sustainably remove an isolated population of *Glossina austeni* Newstead using the integration of insecticide-impregnated screens, insecticide pour-on on livestock and the SIT (Vreysen et al. 2000), and (2) in the Okavango Delta of Botswana, where an isolated population of *Glossina morsitans centralis* (Machado) was sustainably removed using the SAT in combination with traps and targets in the barrier zones (Vreysen et al. 2000; Kgori et al. 2006).

Recently, landscape genetics has established itself as an important area of research/investigation in the field of tsetse fly control (Bouyer et al. 2015; Bouyer and Lancelot 2018; Saarman et al. 2018). This has allowed the identification of potentially isolated tsetse populations, which offers the opportunity of:

1) selecting the most appropriate intervention strategies for stage 1 of the progressive control pathway (PCP) for AAT (Diall et al. 2017), a stepwise approach leading to their reduction, elimination and finally, vector eradication,

2) planning an integrated management approach (stage 2 of the PCP), and

3) the choice of suppression and elimination activities (stages 3 and 4 of the PCP).

Bouyer et al. (2015) developed a friction map between 37 populations of *Glossina palpalis gambiensis* (Robineau-Desvoidy) in different areas of West Africa by iterating linear regression models of genetic distance between the populations and environmental data as predictors and by determining least-cost dispersal paths. The effect of environmental factors on genetic distance was studied using a linear regression model to estimate the relationship between genetic distance and a set of environmental factors. The main variables influencing genetic distance were:

1) the geographic distance,

2) being located within the same river basin or not, and

3) three metrics of habitat fragmentation, namely the patch density, the surface of suitable area, and the maximum distance between the habitat patches (Bouyer et al. 2015).

A density-based clustering algorithm, applied to the Maxent open-source software output (Phillips et al. 2019), identified eight potentially isolated clusters of suitable habitats containing tsetse populations that were located at least 10 km away from the main tsetse belt (Fig. 1) (Bouyer et al. 2015). This is essential for selecting potential target areas that contain isolated tsetse fly populations that could potentially be eradicated in a sustainable way.

Moreover, the population with the highest predicted genetic distance from the main tsetse belt ($P = 0.003$) was located in the Niayes area of Senegal and is the target of an ongoing eradication campaign (Vreysen et al., this volume).

In line with Bouyer et al. (2015)'s approach for identifying isolated tsetse populations, Saarman et al. (2018) developed methods to create a connectivity surface to identify isolated habitat areas reflecting the genetic and ecological connectivity at a spatial scale of interest. By integrating genetic data from 38 samples, remotely sensed environmental data, and hundreds of field-survey observations from northern Uganda, the approach of Saarman et al. allowed the identification of isolated habitat of *Glossina fuscipes fuscipes* Newstead. To identify isolated habitats, the methodological framework (1) first identifies environmental parameters in correlation with genetic differentiation, (2) predicts spatial connectivity using field-survey observations and the most predictive important environmental parameter(s), and (3) overlays the connectivity surface onto a habitat suitability map (Saarman et al. 2018).

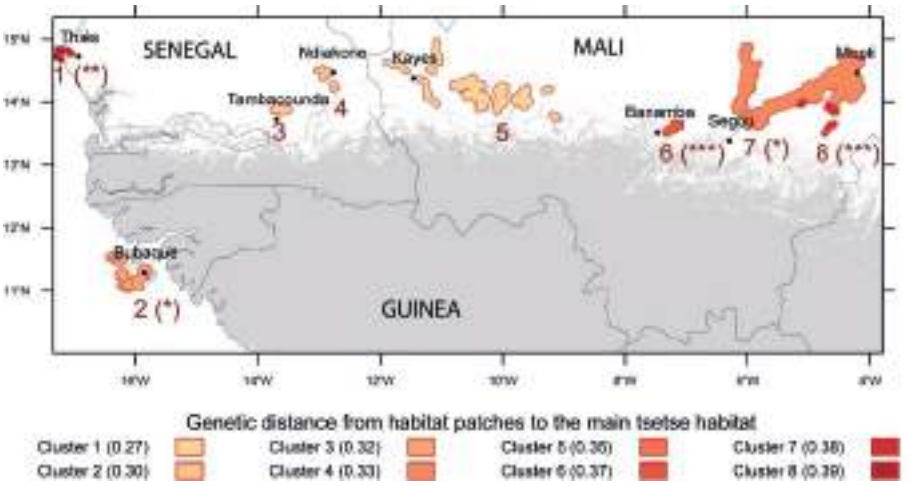

Figure 1. Identification of 8 isolated populations of Glossina palpalis gambiensis *in West Africa. The main tsetse belt predicted by Maxent[1] for a sensitivity of 0.90 is in grey and habitat patches are shown as filled, red shapes. Contours and shapes of isolated patches were defined as 5-km radius buffers around pixels of habitat patches. The genetic distance of these patches to the main tsetse belt (reddish scale) was predicted by the AICc-best regression model along least-cost paths. Star symbols after cluster numbers represent the p values for the friction between the patches and the general habitat: (***) p = 10−3, (**) 10−3 ≤ p < 10−2, (*) 10−2 ≤ p < 5 10−2 (modified from Bouyer et al. 2015).*

[1]The open-source Maxent software is based on the maximum-entropy approach for modelling species niches and distributions (Phillips et al. 2019).

The results from this approach indicate that net photosynthesis is the most powerful predictor of genetic differentiation for *G. f. fuscipes* in northern Uganda. Of the 40 distinct landscape patches of adequate size and distance (purple outlines in Fig. 2a), the resulting connectivity area identified a large, well-connected habitat area in north-western Uganda, as well as 24 plots that contained habitat that was for > 25% considered suitable for *G. f. fuscipes* according to the model (purple outlines in Fig. 2b). These 24 isolated plots were selected as possible candidates to locally create tsetse-free zones and / or testing of new control methods or approaches.

Landscape genetics may also be used to locate areas of high friction where barriers to tsetse dispersal such as insecticide targets or traps are more likely to isolate the target areas.

3.2. Tsetse Distribution Models to Optimize Vector Control

Tsetse distribution models are not only used to map the risk of AAT (Dicko et al. 2015) but are also very useful to optimize tsetse control operations. These models are very useful for selecting priority intervention areas and guiding the management of the vector control operations during all stages of the PCP for addressing AAT (Diall et al. 2017). For example, these models were applied in pilot studies of tsetse control targeting one riverine tsetse species, *G. palpalis gambiensis* in the Niayes area in

Senegal (Dicko et al. 2014), and two savannah species, *G. morsitans morsitans* Westwood and *G. pallidipes* Austin in the Masoka area, mid-Zambezi valley in Zimbabwe (Chikowere et al. 2017).

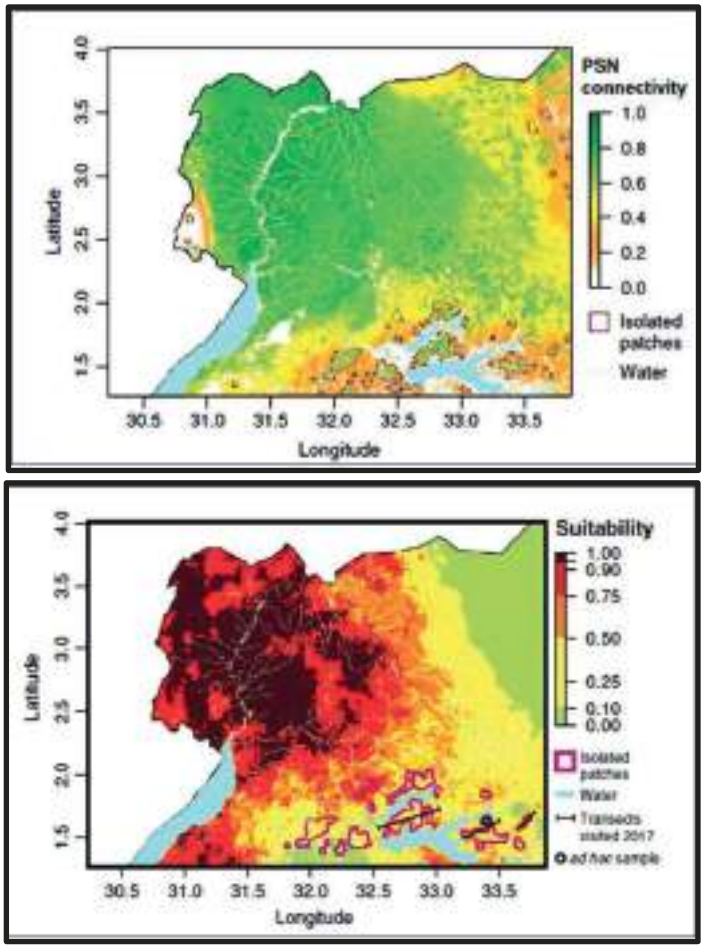

Figure 2a (top). Location of discrete isolated patches in purple and identification of 24 isolated patches of Glossina fuscipes fuscipes *in northern Uganda. A Maxent model was used to produce a connectivity surface, using the environmental variables significantly correlated with genetic differentiation from the previous step and field-survey presence data from 317 traps from northern Uganda.*

Figure 2b (bottom). Habitat suitable for Glossina fuscipes fuscipes *predicted by updating the habitat suitability map obtained with 317 presence data and 12 environmental variables relevant to tsetse ecology by Maxent model. Twenty-four isolated patches identified by the model (purple polygons), the three transects (black lines) used for the field survey, and the location of the tsetse sample from one of the isolated patches used to validate the method (modified from Saarman et al. 2018).*

Using a regularized logistic regression and Maxent, Dicko et al. (2014) compared the probability of presence of *G. palpalis gambiensis* and habitat suitability, respectively. The nature of predictions differed between regularized logistic regression (probability) and Maxent (index). The result provided a better understanding of the relationship between tsetse presence and various environmental parameters as measured by RS. Maxent predicted very well suitable areas considered the most important for an eradication objective, based on an expert-based landscape classification, as some suitable patches can be unoccupied at a certain time and colonized later (Peck 2012), but must nevertheless be included in the target area when applying an AW-IPM strategy (Cecilia et al. 2019).

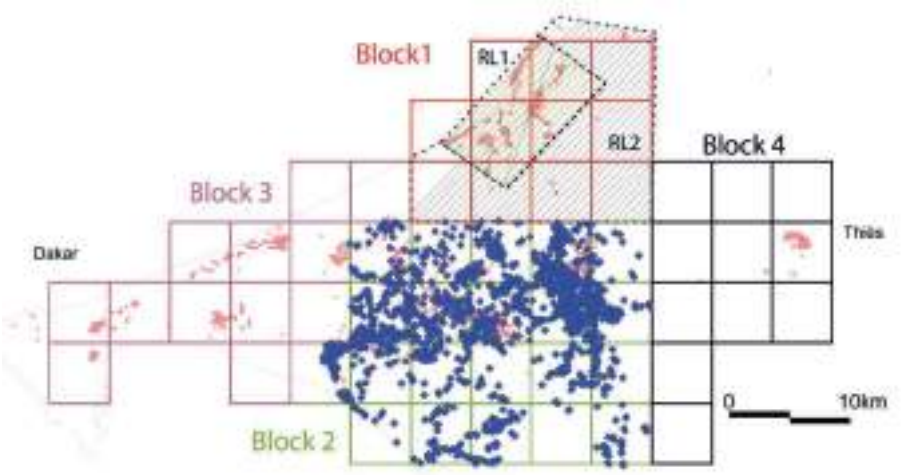

Figure 3. Optimization of the integrated control strategy using model predictions. Maxent model was used to predict the suitable habitats for Glossina palpalis gambiensis *in Senegal. In block 1, the suitable habitats allowed to delimitate two polygons for aerial releases (RL1 and RL2) where the number of sterile males released per km² was adapted to the availability of suitable habitat based on Maxent predictions. In block 1, the green and grey lines present the track flying records of aerial releases on 14th April 2014 in RL1 and 11th April 2014 in RL2 respectively. In block 2, 1,347 insecticide-impregnated traps were deployed from December 2012 to February 2013 in the predicted suitable sites (blue diamonds) to suppress the* Glossina palpalis gambiensis *populations (modified from Dicko et al. 2014).*

Maxent predictions were used throughout the eradication campaign in the Niayes area of Senegal to make the entire operation more efficient in terms of deployment of insecticide-treated targets, release density of sterile males, and the selection of sites to deploy the monitoring traps used for programme evaluation (Fig. 3). Thereby, Maxent predictions allowed optimizing efficiency and reducing the cost of the eradication campaign.

4. CONCLUSIONS

Species distribution modelling and landscape genetics are crucial for planning and optimizing tsetse fly control programmes, especially when sustainable eradication is the selected strategy. Potentially isolated clusters of tsetse fly habitats were identified based on species distribution models and ranked according to their predicted genetic distance to the main tsetse population to locate potential target populations for eradication.

Integrating distribution models and genetic studies in feasibility studies for control operations may also be useful to apply the PCP for controlling AAT, a stepwise approach leading to the suppression, elimination and eradication of vector populations and disease (Diall et al. 2017). Furthermore, distribution models can help improve the efficiency of control activities, leading to a reduction in costs. Finally, these distribution modelling and landscape genetics approaches should be integrated in the future, not only into tsetse control efforts, but might also help in the area-wide management of other pests as well.

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AGENT-BASED SIMULATIONS TO DETERMINE MEDITERRANEAN FRUIT FLY DECLARATION OF ERADICATION FOLLOWING OUTBREAKS: CONCEPTS AND PRACTICAL EXAMPLES

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SUMMARY

Areas of the world that do not have established populations of the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) and other invasive pestivorous Tephritidae are sometimes subject to incursions due to increasing travel and trade. When these occur, control programmes are put in place often including quarantine and additional measures until eradication of the outbreak is declared. A critical practical question that arises is how long to maintain the eradication programme and associated area-wide measures after the last sampling of the invading Mediterranean fruit fly. Current practice is usually to maintain measures and increased monitoring until enough time has passed for three generations of flies without another fly catch; generation times are calculated via thermal unit accumulation ("Degree Day"). A recent alternative or complementary approach is to model the invading population using an Agent-Based Simulation (ABS). This chapter outlines the use of *MEDiterranean fruit Fly Outbreak and Eradication Simulation* (MED-FOES), an ABS implementation aimed at modelling invading Mediterranean fruit fly populations to determine effective duration of quarantine and other eradication measures following the last detection of an incursion. Basic concepts are described, together with a description of major functions and use of thousands of individual simulations to encompass the range of demographic possibilities. Finally, specific examples from Santiago, Chile and California, USA are offered to show how the ABS can provide useful information for programme managers setting eradication programme durations.

Key Words: Invasion biology, incursions, outbreaks, quarantine, eradication, Tephritidae, *Ceratitis capitata*, medfly, monitoring, detections, California, Chile, degree-days, model, modelling, individual-based simulations, multi-agent simulations, MED-FOES

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1. INTRODUCTION

Incursions of the polyphagous pest Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) occur in urban and agricultural areas around the world. The Global Eradication and Response Database (GERDA), although not a complete compilation of all invasions and eradication efforts, currently lists 117 Mediterranean fruit fly incursions and eradication programmes in a wide variety of geographic areas, from the USA to Australia, Chile, Mexico, and New Zealand among others. Responding to each of these outbreaks costs an average of USD 12 million (normalized to 2012 USD) (Kean et al. 2012; Suckling et al. 2016). While this cost is small compared with the estimated damage from a Mediterranean fruit fly establishment in many of these areas (USD 1500 million in the US state of California alone [Siebert and Cooper 1995]), it is still important to optimize responses to incursions. This is because mounting and maintaining an effective response and quarantine is a significant burden and organizational challenge for state and private organizations.

Quarantine measures are often put into place following the detection of an incursion of an invasive pest (FAO 2016a). In the case of Mediterranean fruit fly, quarantine measures involve designating an area around the find where fresh fruits are restricted from leaving. If the area includes commercial host fruit production, then the quarantine measures can result in serious economic losses. Therefore, the period to maintain the quarantine and associated measures against the invading Mediterranean fruit fly population becomes a critical practical question.

Current practice in many parts of the world is based on ISPM 26 (FAO 2016b), such as the protocol used by the California Department of Food and Agriculture (CDFA), whereby the quarantine is suspended when three full generations of the fly have passed without another find; generation times are calculated via thermal unit accumulation (California Code of Regulations 2017). The thermal accumulation development models most often used simply posit that development from egg to adult requires accumulation of a specific amount of heat above a base threshold, measured in units of degree-days (Roltsch et al. 1999). Various calculation methods exist, but all approximate the integral of temperature over time for temperatures above a given base temperature. The simplicity of degree-days calculations is attractive, and thermal accumulation models are widely used with impressive accuracy for predicting developmental timing in many agricultural contexts. However, it may not be entirely appropriate for Mediterranean fruit fly eradication and quarantine duration determination. "Degree-days" is a development model, not a population or eradication model. Moreover, the requirement for three generations of degree-days to pass is difficult to justify theoretically.

An alternative approach to estimating eradication programme length or duration since the last detection is to use an Agent-Based Simulation (ABS; also called "Individual-Based" or "Multi-Agent") to simulate the arrival of *C. capitata* in a new area as an insect invasion (Manoukis and Hoffman 2014). In an ABS, individual flies are described as unique and autonomous, and where they usually interact with each other and their environment at a local level (Railsback and Grimm 2012). An important characteristic is that individual members of the simulation ("agents") are represented independently in computer memory via the simulation software, and these

have behaviours (functions) and characteristics (parameters) that may make them unique. The behaviours and characteristics of the entire system are not explicitly coded by the programme rather they emerge from the interactions and behaviours of the system's constituent agents.

The ABS approach has not received a lot of attention for studies on insect invasions (Crespo-Pérez et al. 2011; although see Vinatier et al. 2011 for an example of an ABS of an agricultural pest), but it has been more widely used to address questions on physiological ecology, foraging networks, ant nest choice, and disease vector dynamics among others (Jackson et al. 2004; Pratt et al. 2005; Almeida et al. 2010; Radchuk et al. 2013).

This chapter is concerned with the concepts, implementation and use of an ABS entitled MED-FOES (*MED-Fly Outbreak and Eradication Simulation*; MED-FOES 2019), that was designed to simulate the invasion, programmatic response to an outbreak, and extirpation of a population of Mediterranean fruit fly in a pest free area (Barclay et al. 2021). The original goal of the ABS and its implementation is to provide an estimate of eradication programme duration that is roughly independent of the values determined by thermal unit accumulation.

In an area-wide context, which means addressing a total pest population within a defined area and not a localised field-by-field approach (Hendrichs et al. 2007), modelling eradication programme lengths is important for several reasons:

First, programmes against Tephritidae tend to encompass large areas, and many of the measures taken following a Mediterranean fruit fly incursion are likewise applied over these same vast areas, such as intense surveillance, restriction of fruit movement and in most cases SIT application. These approaches are not effective in an uncoordinated property-by-property setting.

Second, there are area-wide programme costs to be considered; these can grow to be large if excessively long programmes are implemented, in particular if quarantines and commercial production areas are involved.

Third, eradication efforts are often directly connected to area-wide programmes (Smith 1998; Myers et al. 2000). If eradication programme lengths against Mediterranean fruit fly are too short, there is potential for survival of remnants of the invading population, which would require a new eradication campaign or in a worst case scenario, result in the establishment of the pest species in a free area (Carey 1991; Papadopoulos et al. 2013; McInnis et al. 2017; Shelly et al. 2017).

MED-FOES is based on a modelling framework that simulates the process of Mediterranean fruit fly population extirpation/eradication (Manoukis and Hoffman 2014). A description of the important functions included in the simulation is given in Section 2, with minimal mathematical background. The use of thousands of simulations to understand the range of possible outcomes following an outbreak is the focus of Section 3. In Section 4, two examples of historical incursions are analysed to illustrate the use of MED-FOES for determining eradication programme length. Finally, Section 5, includes broad conclusions and some suggestions for future work. The actual installation and mechanics of using the software are not covered here; the reader is referred to the software manual for these details (MED-FOES 2019).

2. CONCEPTS AND IMPLEMENTATION

2.1. Simulation Description

Mediterranean fruit fly agents in the simulation can be created, and they develop, reproduce and die. The major challenge is to have the agents behave in a biologically realistic manner so that the results of thousands of executions of the simulation can be usefully related to flies in the real world.

Figure 1 gives a graphical representation of the developmental stages and states fly agents in MED-FOES can occupy as well as their connections. During each hour of the simulation, flies may move from one developmental stage to another, or not. They also may go from being alive to being non-reproductive (mated with a sterile male) or dead, when they are removed from the simulation and no longer considered.

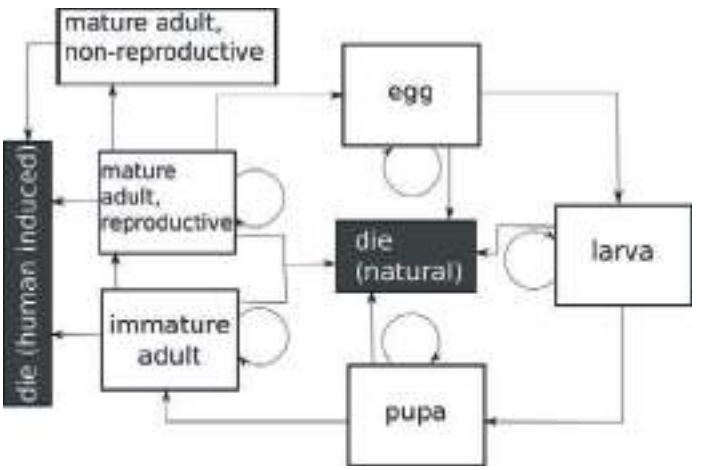

Figure 1. Graphical representation of the developmental stages and states of Mediterranean fruit fly agents in MED-FOES. Arrows indicate changes in stage or state per time step (adapted from Manoukis and Hoffman 2014).

2.2. Initialization

The simulation is initialized based on an estimated number of potential remnant adult female flies in the area of an incursion that has been subject to area-wide application of eradication measures. Given this number, the estimated number of female flies in all other stages is determined based on the expected stable age distribution for the Mediterranean fruit fly from the literature (Carey 1982; Vargas et al. 1997). These flies are then iterated through hourly time steps; for each time step, they may develop, reproduce, die of natural causes or die of human-induced causes.

The current version of MED-FOES includes a simple model of the initial stages of the simulation. There is a time between when a detection that exceed the threshold needed to trigger a response during which full counter-measures are not in place. In general, the number of flies detected until the point of declaration is used to estimate

the initial population size range. During the short period between detection exceeding threshold and counter-measure implementation (2-5 days in many jurisdictions), the population of agents can grow, and no human-induced mortality is considered. At the time R (in days) counter-measures initiate and the population begins its demographic trajectory to eradication. This representation of the early stages is unrealistic, as in real incursions detections may occur asynchronously, trapping densities may be changed in response, and individual counter-measures are applied at different times. However, the simulations are generally not highly sensitive to the length of R or initial population size (Manoukis and Hoffman 2014). Future versions of MED-FOES are planned to have the ability to include more realistic initial stages and the ability to model time varying interventions.

2.3. Hourly Temperatures

The only time-dependent input to the ABS is an hourly air temperature time series. Ideally these data should be acquired from a long-term weather station in close proximity to the outbreak being simulated. Data from airports archived in the Integrated Surface Data repository of the National Oceanic and Atmospheric Administration (NOAA) are often appropriate and methods for processing them have been published (Collier and Manoukis 2017).

Care should be taken to ensure that the temperature data are accurate as the ABS (as well as simpler thermal accumulation models) can be sensitive to biases and errors in the data. For example, a sensor improperly shielded from the sun would tend to report slightly higher temperatures, and the cumulative nature of a thermal accumulation development model would amplify the effect over time. Similarly, spurious very high or very low values produced by sensor errors could lead to erroneous high mortality events in the ABS. Actual temperature data often contain gaps, errors, and a non-uniform sampling rate, and therefore require some 'cleaning' before they can be used.

A cleaning process typically starts with the detection and removal of erroneous outlier values. A large variety of methods exist for outlier removal, but a simple method which works well for temperature time series data is computing the running standard deviation (σ) over a window spanning several days, computing the running median (m) over a several hour window, and rejecting individual temperature values (t) for which the absolute value of $(t- m)/\sigma$ exceeds a predefined threshold.

The next step of cleaning is the identification and filling of gaps in the data. Because of the daily cycle of temperature data, it is appropriate to use different methods to fill small (less than a few hours) and large gaps (a few hours to several days). Small gaps may be filled by simple interpolation, which can also serve to simultaneously resample the data to a true hourly frequency. Large gaps, however, are more appropriately filled by interpolating across observations from the same time of day across days. Gaps larger than several days would likely require special treatment dependent on the particulars of the location and season. If actual hourly temperature data are not available, then an approximation may be inferred from daily minimum and maximum temperatures using established methods such as the one implemented in the "TemperatureEstimator.java programme" distributed with MED-FOES (Reicosky et al. 1989; Campbell and Norman 1997).

2.4. Development

The single factor that determines the probability of stage transition or ovarian maturation (development) for flies in the simulation is temperature. The simulation requires a base developmental temperature T_{min} and time-to-transition K for each of the following transitions: egg to larva, larva to pupa, pupa to adult and adult to mature adult.

Several reports in the literature give the mean time to transition (d) for these stages as affected by a range of constant fixed temperatures (T). The common practice is to regress the developmental rate (= $1/d$) against fixed temperatures, which gives a clear linear relationship for temperatures between about 16 and 30 °C. The linear regression model would then be

$$\frac{1}{d} = a + bT$$

where a is the intercept and b the slope. The parameters required by the simulation are then calculated as follows:

$$T_{min} = -\frac{a}{b}$$

and

$$K = \frac{1}{b}$$

There are two methods available for calculating the probability of stage transition. The simplest is a "uniform" model, where the probability of transition at any given hour is determined only by the temperature during that hour. For each hour, a random number between zero and one is drawn from a uniform distribution for each insect that is not a mature adult. If this number is lower than the developmental rate for that temperature, then a stage transition occurs. The average time for a transition at a given temperature is thus equal to $1/d$.

Note that the "uniform" method described above does not take into account the time each insect has spent in a given stage. Therefore, it is possible (though unlikely for most parameter combinations and realistic temperatures) that an agent could go from being an egg to an adult in a few hours. A more realistic developmental model is the "thermal summation" approach (Fletcher 1989), where each degree above T_{min} for each hour counts towards a required threshold C for stage transition. Variation is included for each individual fly agent when it is created in the form of a variable γ, which is the standard deviation of the variation in development time as a proportion of the development time for each stage. Thus, when

$$C + \gamma < \sum_{t=0}^{i} T_i - T_{min}$$

from the time of insect creation (0) to the current time i, stage transition occurs. Note that the value of C is stage-specific, and γ constant across stages.

2.5. Mortality

There are two ways that death is implemented in the simulation. The simplest approach is to set a fixed stage-specific daily death rate, denoted as M_x. Each hour of the simulation, for each insect at stage x, a random double precision floating point number between 0 and 1 is drawn and if it is lower than $M_x^{1/24}$, then the insect dies. This method is completely temperature-independent and is a useful approximation when the effect of temperature on mortality is unknown.

In the case of the Mediterranean fruit fly reliable data are available on the effect of temperature on daily mortality rates. MED-FOES uses the stage-specific quadratic relationships from Gutierrez and Ponti (2011) for this relationship. When this mode it used, the run-specific parameters M_x are the death rate at the optimum temperature (20–25 ºC) rather than the average mortality per unit time.

Additional mortality on adults is introduced at a given number of days after the simulation starts. The probability of human-induced death is fixed per simulation and the same for mature and immature adults; it represents human-induced mortality as a result of counter measures. Human induced mortality is currently limited to the adult stage, though some control measures, such as fruit stripping, may affect immature flies. After human intervention, it is optionally possible to simulate the effect of trapping separate from mortality induced by other countermeasures. This is discussed in the next Section.

2.6. Trapping

Spatially explicit consideration of the effect of trapping on agent mortality is possible as of MED-FOES version 0.6, using the approach of Manoukis et al. (2014) as implemented in the software "TrapGrid" (Manoukis et al. 2014). For parameters relevant to the Mediterranean fruit fly, see Manoukis et al. (2015). A brief description of the trapping model is given here.

TrapGrid is an implementation of a landscape-level, spatially explicit model of trap networks that incorporates variable attractiveness of traps and a movement model for dispersion. TrapGrid simulates susceptible insect capture by placing traps in a rectangular area. Each trap has a parameter indicating its attractiveness (λ). Using this value, the escape probability for a given insect at a given distance from the trap can be calculated. The calculation of escape probability can be conducted for many points in the trapping grid, yielding an instantaneous estimate of the escape probability. Note that probability of capture is simply 1- {probability of escape}.

We calculate the distance to a given trap as:

$$d = \sqrt{(x_t - x)^2 + (y_t - y)^2},$$

where (x_t, y_t) is the position of the trap and (x, y) is the position of the fly. For $d >= 0$, we use an exponential decay with a logistic ($H(d)$) to model the probability of being captured:

$$p = f(d, \lambda) = \lambda e^{-\lambda d} H(d)$$

where

$$H(d) = \frac{1}{1 + e^{-2\lambda d}}$$

These can be combined, producing the hyperbolic secant:

$$f(d,\lambda) = \frac{2e^{\lambda d}}{1 + e^{2\lambda d}}$$

Fig. 2 shows how the probability of capture changes with distance from the trap given λ. Each trap in a TrapGrid model represents the spatial relationship between distance from a lure-baited trap and probability of capture in the very near future. The parameter λ is the attractiveness of the trap, with smaller values representing a more attractive trap.

One important feature of the capture model used is that, for a given value of λ, $1/\lambda$ is the distance at which there is a 65% chance of capturing a susceptible insect. This allows easy comparison of trap attraction between species and lures. Movement in TrapGrid as used by MED-FOES is simple diffusion (Skellam 1951; Kareiva 1983), through which the probability of capture over time (p_t) is calculated.

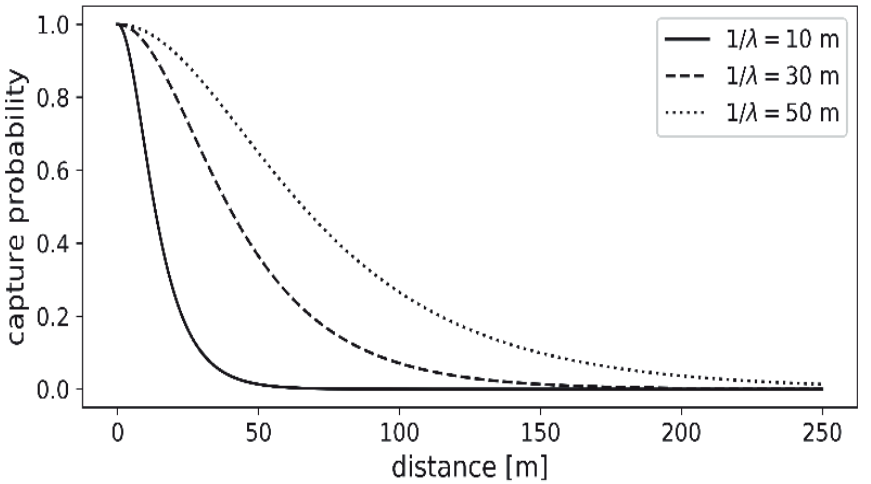

Figure 2. Capture probability for an individual insect versus distance from a trap, where attraction (1/λ) = 10 (solid), 30 (dashed) and 50 (dotted) meters. 1/λ represents the distance at which there is a 0.65 probability of capture (adapted from Manoukis et al. 2014).

In order to make an estimate of p_t, consider the net movement of a fly over the time period in question (usually a day), represented by the parameter D in diffusion models. Estimates of D from the literature are around 1 x 10^4 m^2/day (Corbett and Plant 1993). We can use the net movement per day to model the proportion of the arena space that an individual Mediterranean fruit fly might "experience" per day.

The diffusion in two dimensions is well studied, and has the following form:

$$\frac{dN(x,y,t)}{dt} = D\left[\frac{d^2N}{dx^2} + \frac{d^2N}{dy^2}\right]$$

where N is the population density, t is time, x, y are spatial coordinates and D is the diffusion coefficient. This process produces a bivariate normal distribution of density over time, the probability density function (pdf) of which is the basis of our model of the fly population and its spread over time:

$$g(x,y) = \frac{1}{2\pi\sigma^2} e^{-\frac{1}{2}\left(\frac{x^2}{\sigma^2}+\frac{xy^2}{\sigma^2}\right)}$$

Here, σ is the standard deviation, assuming that

$$\mu = 0,\ \sigma x = \sigma y = \sigma \text{ and that } cor(x, y) = 0.$$

Further description is beyond the scope of this document, but it is important to note that the diffusion parameter D in m^2 per day is the single factor that determines how quickly the simulated flies in TrapGrid will spread.

MED-FOES can run a set of TrapGrid simulations before creating agents. This set of spatial simulations is used to determine the average daily probability of death from trapping given a trapping network following a detection, and then this mortality is scaled to hourly time steps and applied.

2.7. Reproduction

Every mature reproductive Mediterranean fruit fly will oviposit eggs every 24 hours prior to intervention time t_S. In this sense, the simulation only includes females. The mean number of eggs and variance in reproductive output are set by the variables r and r_{var}. After human intervention, reproduction may be curtailed at a set daily rate, which is denoted r_{red}. This variable is included based on modelling of the Sterile Insect Technique (SIT) and its effects on host populations (Knipling 1979).

In the ABS, each mature adult fly that exists at t_S is subject to loss of reproductive ability each day with probability r_{red}. In the case studies below, flies emerging after t_S are assumed to not be able to reproduce. Mating is currently not required for reproduction, so the difficulty in finding a mate that comes with small population sizes is not modelled. In addition, the Allee effect, where there is reduced mean individual fitness in small populations, is also not considered.

3. ANALYSING AN OUTBREAK: MULTIPLE SIMULATIONS

Until this point, we have focused on a single simulation and how biological and population processes are represented. Individual simulations can vary from one to the next, even if the same parameters are used, due to stochasticity (random events) built into the model. However, in order to obtain useful and actionable information on a real-world incursion, a single set of parameters is insufficient because there is uncertainty on the values of critical parameters. Thus, it is necessary to execute a set of simulations, varying parameters between runs, in order to obtain the range of possible outcomes following an incursion. MED-FOES has this functionality built-in via a separate executable programme called “med-foes-p.jar” (MED-FOES 2019).

3.1. Exploring the Parameter Space

MED-FOES includes a large number of parameters that may be set over a range, including aspects of mortality, development, reproduction, and control measures. Even if a fixed number of discrete levels over all these ranges were selected, a full factorial experiment would be computationally prohibitive.

Latin Hypercube Sampling (LHS) is used to select parameter sets through the parameter hyperspace (Blower and Dowlatabadi 1994). The details of LHS are beyond the scope of this document, save that it ensures an even distribution of the combinations of parameters used for individual runs.

The number of simulations executed can be set by the user. A number in the thousands will usually provide sufficient resolution on the range of possible outcomes.

3.2. Summarizing Output

MED-FOES produces a summary output file that is named MED-FOESp_{timestamp}_summary.txt, where {timestamp} is the date and time the programme was run to avoid inadvertently overwriting previous output. This file contains the parameters used as well as results, including the mean, standard deviation, and 25, 50, 95, and 99% quantiles of the length of runs (individual simulations). Since runs nominally start close to the time of last detection and terminate upon eradication of the simulated population, these run length statistics summarize time to eradication across the various parameter sets tested.

Additionally, the mean and standard deviation of number of flies at the end of the simulations are reported and can be used to detect situations where some runs ended without reaching eradication due to an insufficient amount of input temperature data or exceeding the maximum number of flies allowed. Summary figures are produced that give a quick visual summary of the outcomes of the set of simulations (Fig. 3).

A MED-FOESp_{timestamp}_details.txt file is also produced. This is a tab-delimitated table reporting for each run the length of the run in simulated hours, the end condition (eradication, out of temperature data, or maximum flies exceeded), and total number of flies at the end. From this file, arbitrary statistics for the time to eradication can be produced.

Finally, for each individual run a summary and details file is produced under the "runs" directory. The summary file gives the specific parameters used for that particular simulation, in contrast to the MED-FOESp summary file, which gives the ranges sampled by the LHS procedure. It also gives summary results: number of hours simulated, number of flies at the end of the simulation, cumulative number of eggs, and cumulative number of deaths.

The details file is a comma-delimited file with a row for each day of the simulation containing: time, mean temperature, minimum temperature, maximum temperature, cumulative death, cumulative birth, the number of flies in each life stage (egg, larvae, pupae, adult), and total number of flies. The collection of runs details files can be used to produce a wide variety of detailed outputs, such as Figs. 4 and 5 shown in the next Section.

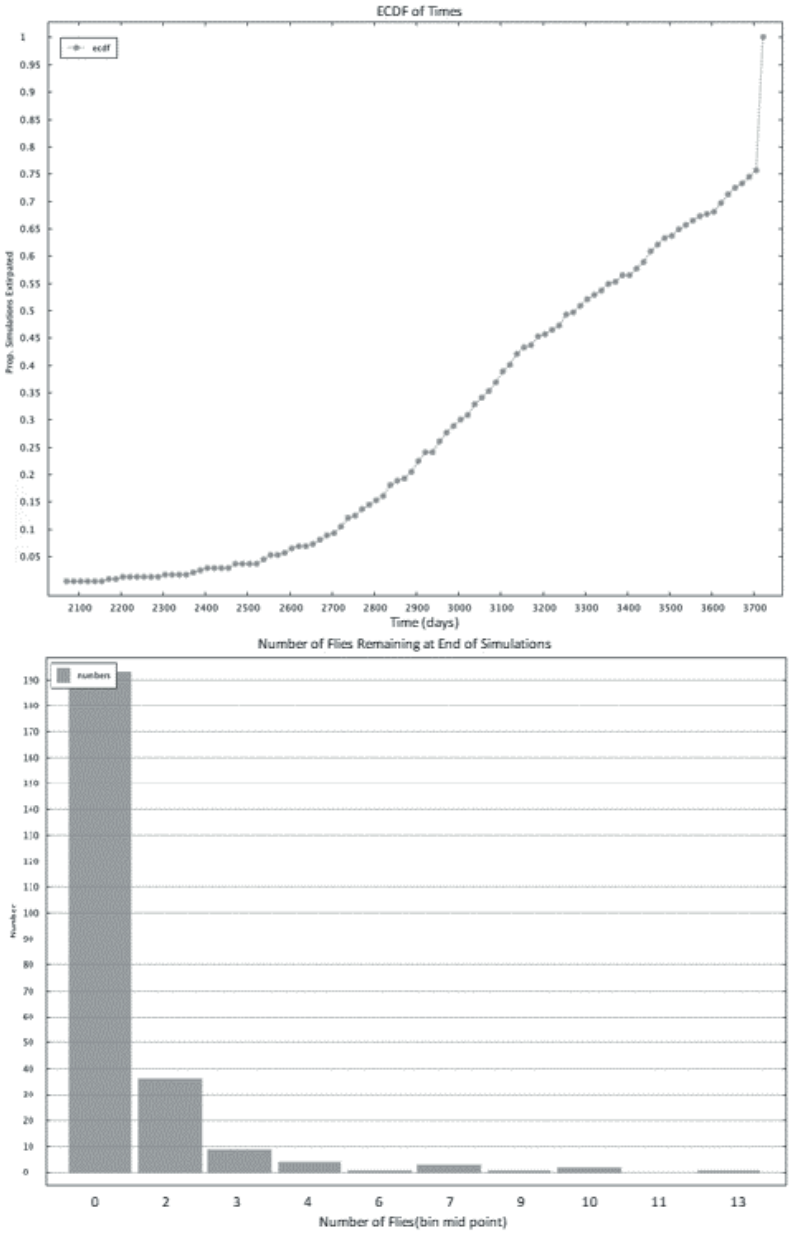

Figure 3. Example of graphical output from MED-FOES showing 250 runs simulating an outbreak of Mediterranean fruit fly in California. Upper panel: Empirical cumulative frequency distribution of the number of simulations showing extirpation over time. Bottom panel: Histogram of the number of surviving agents at the end of the 250 simulations. This simple summary graphic can be used in internal reports without the need of post-simulation data manipulation.

4. PRACTICAL EXAMPLES

4.1. Santiago, Chile

Chile is internationally recognized to be free of tephritids of economic importance, including species in economically important genera *Anastrepha, Bactrocera*, and *Ceratitis*. This is facilitated by Chile's geographic isolation, stemming from the presence of the Pacific Ocean to the West and the Andes mountains in the East, as well as its excellent pest exclusion infrastructure. Despite this isolation, occasionally Mediterranean fruit fly outbreaks are detected in the country, usually via the network of about 14 000 traps.

One such detection occurred in the neighbourhood of Independencia in Santiago, in 2011. The first fly was detected in a Jackson trap baited with trimedlure on 14 October, and the response including quarantine, enhanced trapping, bait spray (GF-120) applications, soil treatment under host trees, and fruit stripping was put in place by 18 October. Another fly was sampled on 18 October in a McPhail trap about 800 m from the first detection; thereafter, no further detections of adults were made. The response programme was concluded on 9 February 2012 after three generations (F_3) of degree-day development as calculated by the Chilean Agricultural and Livestock Service (SAG) using the method of Tassan et al. (1983). This occurred 115 days after the first find.

For the ABS analysis, conducted with version 0.6.2 of MED-FOES, critical parameters that had to be evaluated included: initial population size, reduction in fecundity over time, and hourly temperature data. Starting with the third of these, hourly temperature data were acquired from NOAA's online ISD-Lite dataset derived from the Integrated Surface Database for the weather station at the nearby (approx. 12 km from the outbreak neighbourhood) Comodoro Arturo Merino Benítez International Airport (SCEL). The air temperature data for one year starting on 18-10-2011 (day 0) were extracted and cleaned using the method described previously and saved to a comma separated format (csv) file for MED-FOES to use as input.

The SAG programme did not include SIT for the 2011 outbreak. This might mean that the modeller decides not to include the parameter on fertility reduction per day ("rred"). However, due to the intense fruit stripping (100% in a 400 m radius from each find) plus the soil drenches (not used in California currently), it was estimated that there was probably an effective reduction in the probability of reproduction over a wide range, 0.2-1.0.

Finally, the initial population size was estimated based on the surveillance network in Chile at the time of the outbreak. This consisted of one trimedlure trap per 25 ha and one protein trap per 100 ha, comparable to the California values of one trimedlure and one protein trap per 52 ha. This gives a rough detection sensitivity of 2-3 % of the adult population (Manoukis and Hoffman 2014). Assuming a stable age distribution (from Table 3 in Carey 1982), gives estimated numbers in the other life stages. Though these figures are rough, they are probably sufficient since the model is not very sensitive to initial numbers (Manoukis and Hoffman 2014).

The complete set of parameters used to generate the runs are given below:

T	SCEL_2011-10-18.csv	Hourly temperature data file
Ni	66,100	Initial population size (range)
Ad	29.8,49.7,15.6,1.8,3.1	Initial age distribution (from Carey 1982)
R	4	Days before intervention is implemented
S	0.05,0.15	Daily human-induced mortality (range)
rred	.2,1	Reduction of reproduction (range)
Sai	true	Sterility after intervention
TEL	9.6,12.5,27.27,33.80	Transition parameters, egg to larva (range)
TLP	5.0,10.8,94.50,186.78	Transition parameters, larva to pupa (range)
TPA	9.1,13.8,123.96,169.49	Transition parameters, pupae to adult (range)
TIM	7.9,9.9,58.20,105.71	Transition parameters, adult to sexually mature (range)
Me	0.0198,0.1211	Daily natural mortality of eggs (range)
Ml	0.0068,0.0946	Daily natural mortality of larvae (range)
Mp	0.0016,0.0465	Daily natural mortality of pupae (range)
Ma	0.0245,0.1340	Daily natural mortality of adults (range)
tdm	true	Use temperature dependent mortality
r	5,35	Eggs produced per reproduction event (range)
rvar	3.57	Variance in eggs produced per event
Dm	1	Development model; 0 = uniform, 1 = thermal summation
TuSD	0.05	Variation in thermal unit transition
Tmax	35	Maximum temperature for development
o	Run_2011-10-18	Output directory for results
nR	2500	Total number of simulations to run
nT	20	Number of threads to employ
Mx	500000	Maximum number of flies allowed
seed	4354885	Random number seed
q	true	Suppress progress output to terminal
pr	false	Produce only LHS parameters
plot	false	Generate summary plots

The parameters above when executed on a command line on a computer with a quad-core processor would be invoked roughly as follows (for more details please refer to the programme manual, distributed with MED-FOES):

```
java -jar med-foes-p.jar -T temps_SCEL_2011-10-18.csv -Ni 66,100 -R 4
-S 0.05,0.15 -rred 0.2,1 -Sai true -TEL 9.6,12.5,27.27,33.8 -TPA
9.1,13.8,123.96,169.49 -TIM 7.9,9.9,58.2,105.71 -TLP
5.0,10.8,94.5,186.78 -Me 0.0198,0.1211 -Ma 0.0245,0.134 -Ml
0.0068,0.0946 -Mp 0.0016,0.0465 -tdm true -r 5,35 -rvar 3.57 -Dm 1 -
TuSD 0.05 -Tmax 35 -nR 2500 -nT 4 -Mx 500000 -seed 4354885 -q true -
pr false -plot true -o Run_2011-10-18
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The parameters and ranges above were used to execute 2500 runs. The number of living individual agents per day and mean trend from 100 of these simulations (reduced from 2500 for clarity) is shown in Fig. 4.

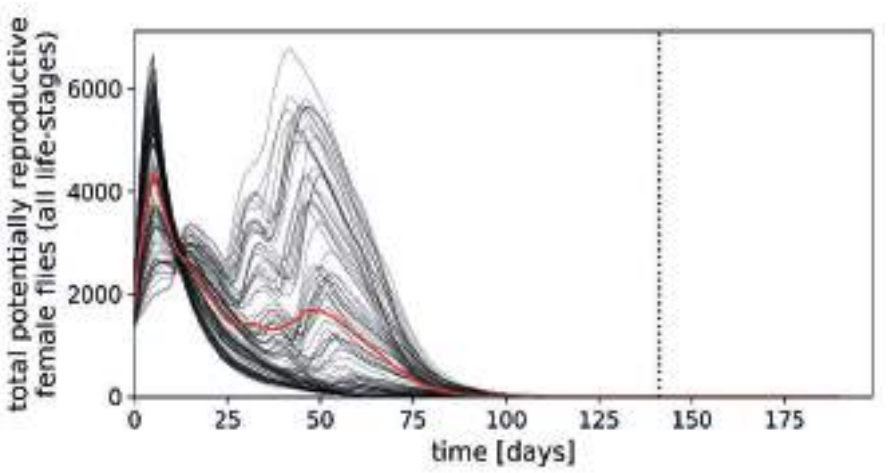

Figure 4. Total number of potentially reproductive female flies including all life stages for 100 simulations, last fly was detected 18-10-2011 (day 0), Santiago, Chile. Mean trend is shown by the red line. The dashed vertical line shows the point at which 95% of the simulations show eradication (141 days).

Fig. 4 shows some interesting characteristics, including parameter sets that seem to lead to increasing population sizes through a second and even third generation. This can be interpreted as a function of favourable temperatures for initial adult survival and reproduction, and for immatures to progress to adulthood from the initial population and from eggs laid before time *R*. Clearly a maximum population size of nearly 7000 individuals is a maximal (and unlikely) scenario, but it is indicated by a small number of the parameter sets simulated; the average time from declaration to eradication was 109 days.

In terms of indicated duration of the quarantine and other control measures, 95% of the simulations showed eradication after 141 days, 18% longer than the F_3 degree-day calculation that led to the actual 115-day control and quarantine period.

It is common for the ABS to predict longer durations than simple thermal accumulation models for outbreaks persisting through the summer in temperate areas. Since the ABS incorporates other factors which may mitigate the rapid generational turnover shown by thermal accumulation in hot weather, or the near halt in development seen in cold weather such as illustrated by the next example, it will often show less extreme seasonal swings than simple degree-day models (Collier and Manoukis 2017).

4.2. Perris, California USA

California has been the site of multiple *C. capitata* detections and outbreaks over the last four decades or so, leading some to propose that the species and other Tephritidae are established there (Carey 1991; Papadopoulos et al. 2013). However, this theory has not been accepted by most phytosanitary experts, internal and external California trading partners, or customers of horticultural commodities (McInnis et al. 2017; Shelly et al. 2017). As in Chile, California maintains a large (>90 000) trapping network for detecting Mediterranean fruit fly incursions. Additionally, since 1996 California has been conducting a preventive sterile male release programme over the high-risk Los Angeles Basin, where most of incursions of this pest are detected.

In December of 2014, Mediterranean fruit fly was detected in the city of Perris, that is located in Riverside County east of Los Angeles. The city is about 27 km outside the zone the preventive release programme covers. The initial detection was a find of two unmated adult females on 10 December 2014 in McPhail traps, which was followed by eight other finds in the same residential area over the next few weeks, including a find of larvae on 14 December 2014. The final find occurred on 29 December 2014. Mitochondrial genotyping indicated the AAAB mitotype consistent with a Central American source.

Eradication efforts started quickly, within one day, and included fruit removal, spinosad foliar bait spraying, and inundative releases of approximately 1.5 million sterile male flies every three or four days. The total quarantine area established was 215 km^2, with the sterile male releases targeting a 33.4 km^2 core area.

The weather station at March Air Reserve Bases (KRIV) is approximately 12 km from the find sites. This station has good data going back to the 1940s, available through NOAA's ISD archive, which allows not only modelling the 2014 outbreak, but also putting it in the context of how a similar outbreak would have progressed if it started on the same day of the year in previous years. Specifically, data from the ISD-lite data from 1950 through 2015 were cleaned as described earlier in this chapter and used to run MED-FOES simulations.

MED-FOES v0.6.2 was run with the same parameters as the Santiago model, except for the initial population (Ni) of 25 to 133 adult females, a delay before the start of SIT releases (R) of 1 day, and the input temperature data. By day 151 after the last fly was detected, 95% of the ABS simulations predicted eradication (Fig. 5). This is more than one month shorter than the quarantine and control period that was actually implemented of 189 days, or 187 days produced by recalculating the degree-day based three generation time (F_3) using the same KRIV temperature data used for the ABS.

In contrast to the ABS results for the 2011 Santiago outbreak, the 2014 Perris, simulations show no evidence of large population sizes after control measures were started. There is a very small increase in number of flies around day 80 for a few of the simulation parameter sets, but the overall character is rapid decline followed by a small population resisting final eradication for a relatively long time. Remembering that any eggs laid after time *R* (counter-measure start) are sterile, only agents that existed before that time could reproduce and account for population growth later in the simulations. Unfavourable environmental conditions for those individuals, leading

to their rapid and early mortality, would cause the difference observed in overall numbers between this case and the Santiago case presented above.

The ABS results indicate eradication occurring significantly earlier than the simple thermal accumulation calculation (151 vs 187 days). This is a common finding for outbreaks covering cold periods in temperate climates, since cold temperatures in the degree-day model just slow development. In the ABS model, however, mortality occurs even with slow development and may even be increased due to particularly cold periods.

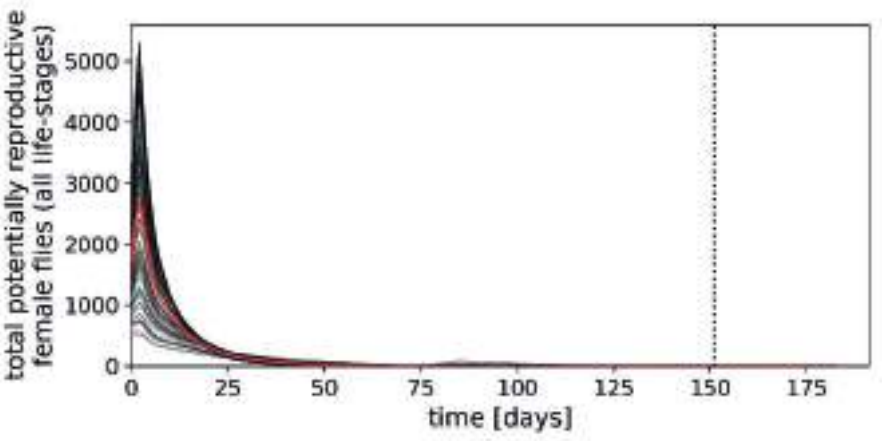

Figure 5. Total number of potentially reproductive female flies including all life stages for 100 simulations, last fly was detected 29-12-2014 (day 0), Perris, California. Mean trend is shown by the red line. The dashed vertical line shows the point at which 95% of the simulations show eradication (151 days).

In addition to simulating the particular outbreak, the ABS can be used to simulate what would happen if the same outbreak occurred at a different time. One application of this is to simulate the outbreak using temperate data from the same date in previous years for either historical context or to produce predictions for ongoing outbreaks. Fig. 6 shows both the time required for 95% of the ABS simulations to reach eradication and the degree-day thermal accumulation-based three generation time (F_3) computed using temperature values from 1950 to 2014. The values for the actual outbreak year of 2014 (filled markers) show that the degree-day calculation is exceptionally short while the ABS is almost the median value of previous years.

5. CONCLUSION

In the context of an area-wide programme aimed at achieving zero pest prevalence, determining the duration of eradication programmes following incursions by invasive tephritid fruit flies is as critical as it is difficult. Critical because failure to eradicate invading fruit flies will lead to increased costs incurred under follow-up programmes once population sizes increase again or are established in a different area, to say

nothing of the costs of establishment in areas where they are not present (Siebert and Cooper 1995). In addition to these risks from control/quarantine periods that are too short, it is also important not to set overly long periods as these could be unnecessarily burdensome to producers and also lead to excessive programme costs and losses. The difficulty, however, lies in estimating the size of a possible remnant population of flies that contains so few detectable individuals (nominally adults responsive to the lure being used in the trapping array) that it is unlikely to catch any initially (Carey et al. 2017; McInnis et al. 2017).

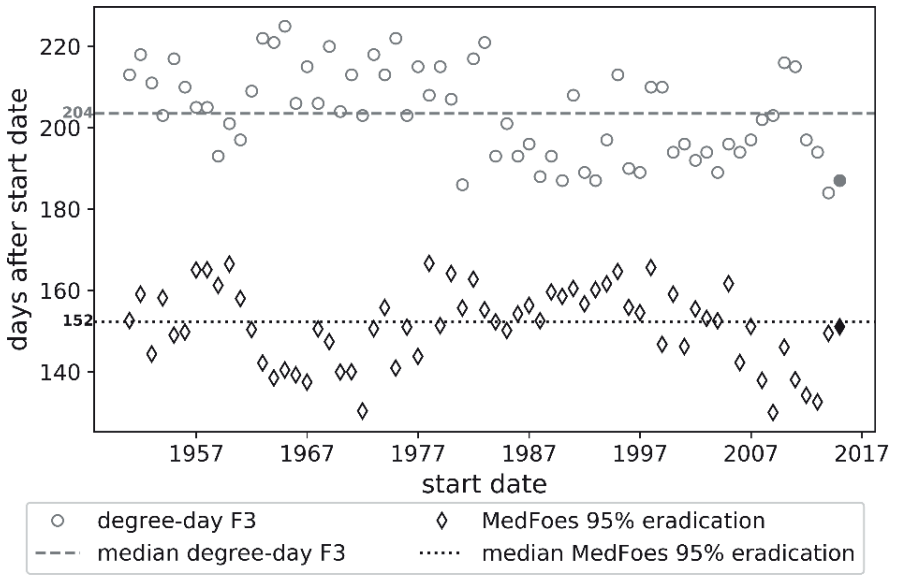

Figure 6. Times required for 95% of the ABS simulations to reach eradication in comparison to the three generation times (F_3) based on the degree-day thermal accumulation computed for hypothetical Perris, CA outbreaks staring on 29 December for the years 1950 through 2014. The actual outbreak in 2014 is shown by filled markers. Degree-day based values are circles, and ABS based values are diamonds. Dashed lines are medians.

Though the task is difficult, the established method of using simple thermal accumulation models to guide control activity durations and ending quarantines after the equivalent of three generations of degree-day development have elapsed without another find seems to work well in practice in many places around the world, including Chile and California as described here, though there are limits in more temperate areas (Collier and Manoukis 2017).

Agent-Based Simulations, as modelled in MED-FOES, can be a useful complement for programme managers and state entities as they provide a semi-independent estimate of the duration that control and quarantine activities should continue after the detection of the last fly find. The software is relatively easy to use and with increasing computational power in multi-core desktop computers it is feasible to run a large number of simulations in a relatively short time.

It is important to stress also the drawbacks of using ABS methods in general. These include the need for range estimates of many constituent parameters. In the case of Mediterranean fruit fly over 100 years of research on development, reproduction and mortality of this species is helpful, but some details remain poorly resolved, especially under natural conditions. A second general problem with individual-based methods is that simulating combinations of parameters to exhaustively test the range of possible outcomes can be computationally expensive. Here, again, current computer systems are powerful enough to mitigate this issue, but this problem can increase as models become more complex. A final point here is that the stochastic nature of the simulation and variable output necessitates statistical methods to handle results, and the mechanisms that drive outcomes may not be immediately clear.

One possible approach that integrates the current approach and the ABS would be to set the initial eradication programme duration following the last detection after an outbreak using the three-generation calculation on historical temperatures (already done in at least Florida and California USA, Gilbert et al. 2013), and then update with both degree-day calculations using measured hourly temperature data and with ABS simulations. The combination of both estimates will give improved insight to the outbreak dynamics, might suggest programmatic responses of effort modulation, and increase confidence in declarations of eradication.

MED-FOES instantiates a relatively simple model and therefore has several notable weaknesses. Control measures are simulated as constant average effects instead of time-varying, while eradication programmes typically incorporate discrete high-intensity efforts such as fruit removal, which will have different impacts depending on the age structure of the population. Additionally, quarantines are held for a period of time (at least one degree-day generation for *C. capitata* in California) after the end of suppression efforts during which intensive trapping continues. Finally, incorporating information on host plant availability, when known, would likely have significant effects. However, a major strength of the ABS approach is that all of these factors and more could be incorporated into the model in a straight-forward manner given the relevant input data.

Future developments for MED-FOES aimed at facilitating declaration of eradication after outbreaks include improved reporting incorporating analysis of historical temperature profiles. The analysis of temperatures in past years will be helpful for tracking anomalously long or short quarantine/control periods after the last detection, again increasing confidence in lengths. The flexibility and extensibility of the ABS framework also allows other applications of this model to address questions around the invasion biology of *C. capitata.*

One of the new applications of MED-FOES currently being explored is to estimate the potential growth rate of Mediterranean fruit fly populations in different areas and at different times of the year. This would yield results that could be compared to those from standard climatic suitability methods such as CLIMEX and Maxent (Webber et al. 2011). The specificity of MED-FOES to the biology of Mediterranean fruit fly could serve to refine the more general spatial and temporal analysis of patterns of detections and outbreaks when compared to the standard approaches based on environmental suitability modelling, especially when combined with incursion risk data.

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REAL-TIME INSECT DETECTION AND MONITORING: BREAKING BARRIERS TO AREA-WIDE INTEGRATED MANAGEMENT OF INSECT PESTS

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SUMMARY

Area-wide integrated pest management (AW-IPM) of insect pests relies on surveillance and communication to estimate wild population size, guide targeted control, and determine the effectiveness of any pest control action. However, knowing where and when pests arrive in real-time, communicating the information quickly, and delivering insect pest control in a coordinated manner are potential barriers to achieving area-wide management. Agricultural technology is creating opportunities to remove these barriers, which in turn will facilitate the adoption of AW-IPM. Technology advances in insect surveillance (detection and monitoring), data flow and information communication are being realized, and increasingly becoming commercially available. This technological change is largely being driven by macro-economic trends of increased cost of labour, international agricultural trade and shifting consumer demands, and a confluence of new hardware technologies that free computation from the desktop. As professionals and practitioners of pest management, there is an opportunity to shape technological solutions to remove barriers to AW-IPM, and to achieve sustainable pest management across commodities and pests. Yet, the success of the technological solution and its area-wide implementation will depend on the way that we think about the problem (innovation), and the solutions (engineering).

Key Words: Insect remote monitoring, automated surveillance, pest trapping, data flow, data visualisation, agricultural technology, innovation, fruit flies, Tephritidae

1. INTRODUCTION

Pests of global significance regularly cause economic loss due to their transboundary nature and because they are difficult to manage. They are often highly mobile, fail to recognize property boundaries, reproduce rapidly, and require labour-intensive actions to monitor their arrival and to control their populations. Coordinated, well-

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timed delivery of pest control options is often seen as a solution. This approach is referred to as area-wide integrated pest management (AW-IPM); essentially the control of total target pest populations within a delimited area (Klassen and Vreysen 2021). Successful AW-IPM of insect pests requires often highly coordinated effort, involves a regulatory framework, and integrates multiple pest control tactics (Hendrichs et al. 2007).

Evidence of success of the coordinated actions of many farmers was first shown theoretically by Levins (1969). He introduced the concept of metapopulations and distinguished between the dynamics of a single population and a set of local populations. Levin's theoretical model was motivated by, and applied to, a pest control situation over a large region, in which local populations would fluctuate in asynchrony and generations overlap. The output of the model showed that control measures should be applied synchronously throughout to achieve suppression. Many practical examples, successful and unsuccessful, of pest control applied at an area-wide basis have followed (Vreysen et al. 2007; Schellhorn et al. 2015). However, there are many potential barriers to efficient and sustainable AW-IPM such as the inability to know where and when pests arrive, communicating the information on time, and delivering pest control as a rapid response in a coordinated manner.

The AW-IPM relies on three key elements: a) accurate estimates of the pest population across a contiguous area of production and continuously over time, b) efficient communication of the population estimates to pest control managers, and c) dynamic coordination and delivery of the management action to suppress or eradicate the pests. Population estimates are generated by regular inspection of traps, plants, or sentinel animals at fixed or random locations (Southwood 1978). Weekly or fortnightly, the presence of pests or their absence is usually recorded on data sheets in the field, and later entered into an electronic database. Depending on the end user, the information may be communicated within 48 hours of weekly / fortnightly monitoring (e.g. crop agronomists to farm manager) or accumulated in a database for record management needed for historical reflection or a random audit (e.g. government biosecurity).

However, for each of these three elements there are several challenges. Manual inspection of traps is labour-intensive, tedious, and causes delays between insect detection and communication to pest managers. The sampling schedule of 7-21 days allows for pest population persistence and increase without intervention. Communicating insect data in a timely manner from across a contiguous area is unrealistic, unless there is an extensive workforce collecting the insect data and providing it quickly to the pest managers (Enkerlin et al. 2017). Once the end users have the data, delivering pest control in a coordinated manner, which can achieve area-wide suppression, is often logistically challenging, and costly. Up to now, many of these challenges have been mitigated by conducting each element with a centrally organized programme almost exclusively led by governments (Kean et al. 2019). This is certainly true in the case of those integrating the delivery of sterile insects (Dyck et al. 2021).

Pest population estimates, communication, and coordination are organized centrally with many stakeholder participants, which is seen as essential to the success of area-wide programmes, including those with a Sterile Insect Technique (SIT) component. Compared to the number of pests of global significance, there are relatively few examples of AW-IPM of endemic or established pests (Vreysen et al. 2007) because of these barriers, but that is changing. Barriers to more efficient:

a) pest detection, delimitation and monitoring,
b) data flow,
c) information communication, and
d) coordination of pest control,

are being removed through innovations in digital agriculture technology.

Increasingly the inputs and outputs in primary productions systems are being tracked, measured and analysed by automated or partially automated systems. These new technologies increase efficiencies, reduce labour costs and speed up capabilities for decision making. The pressure to continue developing these technologies is driven by global food and fibre demand, market access and traceability issues, and the approaching horizon of resource limitation (FAO 2017). The result of this is that more and more farms are connected and networked, and that data flow is moving away from the notebook and into digital information systems. This is critical in the case of early detection and rapid response to invasive species.

Venture capital investment in agricultural technology is on an exponential growth trajectory. For example, investment in early stage companies in Australia, Canada, Israel, and New Zealand has increased from USD 5.8 million in 2010 to USD 89.5 million in 2017. Growth areas include crop protection and input management, precision agriculture and imagery (Finistere Ventures 2018).

Technology is providing opportunities to achieve greater efficiencies for on-farm- and AW-IPM. Advances have been made in data flow, real-time insect surveillance (detection and monitoring), information communication, and mating disruption by using automated pheromone dispensers. Over that last decade, automation of pest surveillance and taxonomic identification, especially for tephritid fruit flies, has emerged in research institutions and the commercial market (Jiang et al. 2008; Liu et al. 2009; Faria et al. 2014; Philimis 2015; Doitsidis et al. 2017; Goldshtein et al. 2017; Potamitis et al. 2017a, 2017b; Shaked et al. 2018). However, in order for the implementation and adoption of area-wide automated insect surveillance to occur, two spheres will need to align: the way we think about the problem (innovation), and the way we solve the problem (engineering). How we bring these two spheres together will be critical to achieving efficient detection of exotic pest incursions, monitoring of endemic populations, and their control.

Here we focus on *why* we want to bring innovation and engineering together to achieve efficient and effective AW-IPM, *what* methodologies are currently on the market, and *how* current and future technological solutions will contribute to more efficient and effective pest suppression and eradication.

2. THE PROBLEM AND THE SOLUTION

2.1. Significant Barriers to Area-wide Suppression

> *"Innovation in all aspects of modern life is seen as a socio-economic cure for many of the troubles of modern societies"* (Ferguson et al. 2014).

One of the great troubles of modern society is achieving environmentally acceptable and economically sustainable pest management of food and fibre crops. This is especially true for managing highly mobile, invasive insect pests. The AW-IPM of economically important pests is viewed as a promising solution. Yet, to achieve area-wide pest suppression one needs to overcome significant potential barriers including:

a) support from many stakeholders, the community and public (including standardised approaches/methodologies across diverse stakeholder groups),

b) knowing where and when pests show up, and

c) the dynamics of pest populations in target landscapes that are heterogeneous in space and time.

Technological innovation offers solutions to overcome these challenges, making AW-IPM accessible for the management of numerous pests by communities and grower groups, and as well as increasing the efficiency and effectiveness of programmes that integrate the SIT. Logistical barriers can be reduced and eliminated to improve insect detection and monitoring, data flow, communication, and coordination of area-wide pest control.

2.2. The Innovative Solutions to Barriers of Efficient and Effective AW-IPM

The methodologies for in-field data collection of insect populations have barely changed since estimates began and these are well captured in classic references such as Southwood's 1978 "Ecological Methods"; visual counts, sweep nets, pitfall traps, destructive sampling of fruits, and lure-based insect traps are the standard. Generating population density estimates using these approaches is tedious, laborious, and restricted to snapshots in time and space. The data are collected in the field, then returned to the laboratory for manual entry into spreadsheets for later data checking and analysis of trends; a slow in-flow of data, which means a slow out-flow of information.

One of the most recent advances to speed up data flow is in-field electronic data entry. As of 2017 there have been approximately 30 downloadable mobile scouting apps for in-field data collection, a large proportion of which can be used to collect arthropod population data (Hopkins 2017). This technology is increasingly being used across the public and private sectors. Tablet or smartphone applications allow government officers and commercial pest control advisers to enter geo-referenced information at the location of data collection. By scanning a barcode on an insect trap, or geo-referencing a field scout's location, field staff can enter the insect count data on the device, which is then uploaded on a server in an easily readable format such as an Excel spreadsheet.

End users now have the option to use open source software to tailor data collection applications. A widely used example is the Open Data Kit (ODK) Project developed at The University of Washington (users range from individual researchers to Google and the World Health Organization). The project provides tools for a community of users to both create data collection apps and to contribute to the development of the software code. Blogs and forums facilitate an iterative process whereby ODK is continually updated by the feedback loop of users and developers actively engaging (ODK 2018). Fit-for-purpose packages are increasingly available as semi-commercial and commercial applications like those developed and used by different government agencies for fruit fly in-field data collection / communication (Table 1).

Table 1. Examples of end-user dashboards for in-field electronic pest data collection

Name	Location	Application	Target
CalTraps http://caltrap-info.com/faqs	Los Angeles County, USA originally, now covering most California counties	Pest detection data management applications	Tephritid fruit flies
Trapbase https://www.agriculture.vic.gov.au	Victoria, Australia	A Victorian Government online database for recording fruit fly trap locations, monitoring and mapping of fruit fly detections	Tephritid fruit flies
OpenScout www.dtn.com	Indiana, USA, DTN, formerly Spensa Technologies	Field scouting app; allows end user to select insects from a customizable list	Agronomic platform with scouting for insect pests and decision making
AgWorld Scout https://www.farms.com/agriculture-apps/crops//agworld-scout	Perth, Western Australia	In-field assessment to monitor crop health and pest pressure	Custom list of insects
Scoutpro www.scoutpro.org	Urbandale, Iowa, USA	Scouting platform with built-in ID keys	Developed for soybean, corn and wheat
Farm Dog https://farmdog.ag	Salinas, California, USA	Pest and disease management software	Custom list of insects
Koppert iPM Scout app www.koppertipm.com	Berkel en Rodenrijs, The Netherlands	Pest and disease scouting	Customised template
CropScout https://www.agric.wa.gov.au /apps/cropscout	Western Australian Government	Pest counts relative to spray thresholds	Canola aphid

In-field electronic pest data collection applications improve data flow and remove delays caused by manual data entry of a paper-based collection system, which then has to be entered into an electronic spreadsheet, collated, checked for errors, and presented in a meaningful visualization, which is rarely spatially explicit. In turn these new applications can improve communication among pest control managers and other stakeholders; pest location and density can be visualized spatially across an area allowing for a targeted response and review.

However, two significant barriers remain – the spatial and temporal resolution of the information is coarse due to the limitations of 'boots' on-the-ground, both in terms of the number of locations that can be sampled, and the frequency at which information can be collected. Data interpolation has been the primary means of getting around this issue. Yet, the benefit of the visualization of interpolated data does not translate into location-specific pest management. The scale of interpolated data is too coarse to achieve more targeted insecticide or biocide application. Automated insect detection and monitoring has the potential to address this problem effectively.

3. THINKING ABOUT THE PROBLEM

Thorough consideration of the barriers to pest detection and monitoring is needed in order to propose and develop the most useful automated solutions and process for adoption. An engineering solution alone, without the full context of an area-wide management programme, is unlikely to have the anticipated uptake and impact. As one example, trapping grids for pest detection are labour-intensive, therefore a simple solution would be to automate an insect trap to reduce the needs for field visits and labour. However, the result may be a reduced workforce with limited ability to respond rapidly when borders are breached, and an exotic pest invades.

3.1. Detecting Rare Events and Early Incursions

Following a detection of fruit flies in a fruit fly free zone, there is a requirement to move quickly and identify the area infested and the area that has remained free from fruit fly. This can be achieved by rapidly deploying traps at a higher density (10-fold increase) and checking the traps more frequently. Such an event often requires additional staff, and the process continues until the area is declared pest-free. Arguably, the problem is the low probability to detect a rare event or early incursion, the challenge to quickly delimit the infested area and deliver control of the infestation, and ultimately to provide sufficient evidence of pest absence.

Solving the problem of detecting rare events and early incursions for a rapid response can minimize the size and duration of the management response, the cost, and the amount of time that markets are closed (Suckling et al. 2016). As two small examples, approximately USD 720 000 / week was spent to tackle the Queensland fruit fly, *Bactrocera tryoni* (Froggatt) incursion into the pest-free state of northern Tasmania, Australia in autumn 2018 (Beavis 2018). In Miami-Dade County Florida, USA, the authorities spent USD 3.5 million in a few months eliminating an outbreak of *Bactrocera dorsalis* (Hendel), and an estimate of ~USD 25 million was incurred

from cost and losses (Alvarez et al. 2016). An outbreak of *B. tryoni* in New Zealand in 2015 cost USD 9.72 million (BBC 2015).

These examples highlight that an innovative solution for biosecurity is a pest surveillance system that increases the probability of detection of rare events and early incursions; a solution that provides greater spatial and temporal resolution. This would allow a rapid response to contain the geographic extent of the outbreak, and to have confidence of area-freedom during and after control. Technological innovations that can quickly provide solutions to costly problems, such as these, are likely to have tremendous positive impact.

Insect detection solutions that are fast and easy to deploy, provide real-time data flow, and are cost-effective, will be even more significant for managing pests that are endemic and widespread. Pest control solutions across commodities mostly rely on the 'sample, spray and pray' approach, with sampling often being *ad hoc* (Zalucki et al. 2009). Searching for pests in orchards and crops requires training for correct identification, can be imprecise (how much searching is required to make an informed decision), is tedious and tiring (long periods of time on hands and knees looking at plants) and those who are hired to complete that task can be incentivized from sales of insecticide, not from not spraying. Advocates of IPM promote sustainable practices, but limited adoption of AW-IPM is the reality. The 'sample, spray and pray' principle is standard, and will remain so until practical solutions for insect monitoring and communication are provided. Technological innovation can play a role in breaking down the challenges of poor information about pest numbers, pest locations and effectiveness of insecticide sprays.

3.2. Trapping Guidelines

Beyond the technical challenges of automating insect surveillance, consideration must be given to the staffing needs and workplace culture when changes are made to long standing surveillance practices. For major insect pests, especially those that are barriers to trade such as fruit flies, insect trapping guidelines are well established, and harmonized based on the trapping objective, e.g. detecting, delimiting, monitoring, and the desired pest control outcome (FAO/IAEA 2018; ISPM 2018). These recent trapping guidelines are comprehensive and provide a level of detail rarely available for other insect pests.

One of the more challenging aspects of trapping guidelines is trapping density, its dynamic, and changes according to the survey objectives, and the pest species. Lure-based insect traps provide relative estimates expressed as numbers per unit effort and are dependent on many factors. In general, the probability of detecting an individual insect is based on the sampling effort (the number of traps deployed), the size of the target insect population, the activity of the insects, and the insect attractant and trapping device efficiency (Vreysen 2021; FAO/IAEA 2018).

3.3. Lure-based Trapping Methods

Lure-based trapping methods are used for many of the major global insect pests for monitoring endemic populations, and for detecting exotic pest incursions, such as

major fruit fly species (e.g. oriental fruit fly, Queensland fruit fly, Mediterranean fruit fly (*Ceratitis capitata* (Wiedemann)), other invasive insects (e.g. Japanese beetle, *Popillia japonica* Newman; tropical gypsy moth, *Lymantria pelospila* Turner; Asian long-horned beetle, *Anoplophora glabripennis* (Motschulsky); Khapra beetle, *Trogoderma granarium* Everts); major endemic pests of field crops (e.g. noctuid armyworm, *Pseudaletia unipuncta* (Haworth); fall armyworm, *Spodoptera frugiperda* (Smith); corn earworm, *Helicoverpa zea* (Boddie)), and pome fruit pests (codling moth, *Cydia pomonella* (L.); oriental fruit moth, *Grapholita molesta* (Busck)*;* oblique-banded leafroller, *Choristoneura rosaceana* (Harris)). These lure-based trapping methods also provide the cornerstone of many SIT programmes, both for delivery of sterile insects (determining release rates) and demonstrating efficacy of control of wild populations.

Lure-based traps estimate the relative density, unless the target's physiological response to the attractant is quantified (Taylor 2018). Relative estimates are expressed as numbers per unit effort and are advantageous over visual estimates by saving time (checking a point location is quicker than random searching of plants and animals), thus increasing efficiency of detection. Lure-based trapping, to generate tephritid population estimates, has limitations in that it is primarily males that are lured, and trapping efficacy can be influenced by various factors, including environmental differences, demographic factors, and the behaviour and physiological state of the individual (Taylor 2018).

Nevertheless, lure-based trapping has become highly specialized and efficient for tephritids (FAO/IAEA 2018). Trapping continues to provide a reliable and easy to use methodology for surveying pests, but also represents a costly and inefficient component of any insect survey programme, be it for government biosecurity or pest management. As an example, the trapping component of SIT-based AW-IPM programmes for Mediterranean fruit fly is estimated to represent 18-25% of programme costs, which is only partially due to the sorting of captured wild from sterile flies (Enkerlin et al. 1996; FAO/IAEA 2018). The trapping cost can be further reduced with automation, and with the technology of today it may even support SIT programmes by helping to prioritise workflow of staff.

4. SOLVING THE PROBLEM

Freeing computation from the desktop has been a key driver that has enabled the development of innovative solutions to automate insect monitoring and detection.

4.1. Automated Insect Monitoring and Communication Technology

Thus far, the majority of solutions include camera/s focused on dead insects caught in the bottom of traps or stuck to sticky cards (Table 2). As with manual traps, all of the automated solutions include off-the-shelf pheromones or attractants of the target insects. The camera takes pictures at fixed intervals (e.g. Trapview, Semios), and the software displays the images for the end user to confirm. For some systems, the confirmation process feeds back into the software as a component of the machine learning algorithm for improved automated diagnostics (e.g. Trapview).

Optical photosensors are being used to discriminate wing-beat frequency of insects entering a trap (e.g. Farmsense and AgroPestAlert). A company established by the authors of this paper use a type of capacitance sensing, similar to a behavioural fingerprint of insects, which detects and discriminates an insect as it enters the trap and delivers the information to mobile app in real-time (Table 2).

Table 2. Examples of automated insect monitoring and communication technology

Company Name	Location	Type	Target	Product Stage
DTN (formerly Spensa Tech)	Purdue, Indiana, USA	Sentinel; camera-fitted trap	Lepidopterap ests of pome fruit	Acquired by DTN April 2018; Commercial www.dtn.com
DTN (formerly Spensa Tech)	Purdue, Indiana, USA	Z-Trap; electromagnetic current inside delta trap	Lepidopterap ests of pome fruit	Acquired by DTN April 2018; Commercial www.dtn.com
TrapView	Hrusevje, Slovenia	Camera-fitted traps in various designs	Lepidoptera and Diptera	Commercial www.trapview.com
SnapTrap	Victoria, Australia	Camera-fitted traps	Fruit fly	Commercial www.snaptrap.com
SemiosBio Technologies Inc.	Vancouver, British Columbia, Canada	Variable rate mating disruption and automated pest camera trap	Lepidopterap ests of fruit and nuts; CA red scale	Commercial www.semios.com
iScout (Pessl Instruments)	Graz, Austria	Camera-fitted sticky trap	Non-specific	Commercial http://www.pesslinstru ments.com/
RapidAIM Pty Ltd.	Brisbane, Queensland, Australia	Novel capacitance-type sensor	Fruit fly for 1st product	Commercial www.rapidaim.io
AgroPestAlert	Tudela, Spain	Photosensor – wing-beat frequency	Non-specific	Prototype http://agropestalert.com /
Farmsence Inc.	Riverside, CA, USA	Optical – wing-beat frequency	Lepidoptera	Prototype www.farmsense.io

Electromagnetic current is used by Spensa Tech for monitoring lepidopteran orchard pests. The current from the 'Z-trap' surrounds and kills the insect, and the amount of current provides an indication of the insect size, as a surrogate for the insect's identity.

There are key considerations when adopting automated monitoring and communication technology, and these will differ depending on the objectives of the programme, and of the end users. However, general criteria include the reliability of detection, reliability of communicating the detection, efficacy of insect capture compared to an industry standard, efficiency compared to manual trapping, and the added value, for example, how real-time data flow allows for rapid response, which ultimately reduces costs of managing outbreaks, and minimizes disruption to trade.

Technology is rarely neutral, and the expectations of the benefits will need to align with the problem that is being solved. For example, if the expectation is that automation will replace humans, then it's unlikely that automation will ever be developed at a level that is cost-effective. However, if the expectation is that automation is providing better information for more informed pest management decisions, improving workflow, and reducing the harm from exotic pest incursions, then there are already technologies that can advance pest detection and monitoring (Table 2). Automated systems are already improving data flow into geographic information system (GIS) databases, in turn allowing real-time visualisation for managers and stakeholders of insect population hot spots and areas under control, and ultimately providing tighter feedback in detection, communication, control application and validation (Fig. 1).

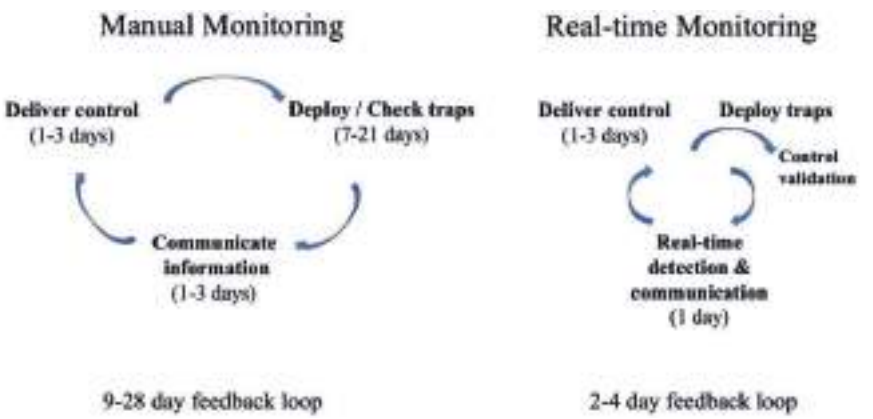

Figure 1. Feedback loop comparing manual and real-time monitoring.

4.2. Coordinating Data Flow, Control and Evaluation

For many decades, theory has demonstrated that for pest population control measures to be effective within AW-IPM, synchronicity at temporal and spatial scales is paramount (Levins 1969; Ives and Settle 1997). Surveillance methodology and strategy, if it is to be effective within AW-IPM, needs also to be synchronous across insect present and potential distribution areas. Some of the pitfalls of manual monitoring are the variations in methodology between stakeholders, as well as human error, and the large and complex chain that data progress through from field collection to management action.

Digitising and standardising data flow at all levels may greatly reduce the loss of data fidelity and allow for the level of dynamism that is required in large complex systems. Examples of where this model has been effectively rolled out at large scales are found within clinical data systems. The transition of medical data records from paper to personal computers to medical data collection apps has accelerated in the past 10 years (Zhang et al. 2017). So much so that clinician end user demand has driven the standardisation of digital health record-keeping at all levels of the data supply chain (FDA 2018).

Empirical examples have shown the benefit of synchronicity in pest control and include coordinated timing of insecticide application (Smith 1998; Lloyd et al. 2010), coordinated growing of trap crops (Sequeira 2001), area-wide release of sterile insects (Hendrichs et al. 1995), coordinated orchard hygiene (Lloyd et al. 2010), and sanitation methods to destroy a life stage, such as pupae in soil, to achieve area-wide reduction in pest populations (Duffield 2004; Lloyd et al. 2008). Some of these examples achieve coordination by default due to the narrow window of suitability of the action, e.g. as defined by the license for growing genetically modified cotton in Australia. However, area-wide data collection, data flow, and coordinating pest management of insecticide/biocide/biological application (sterile insects and natural enemies) has been logistically impossible without a central coordinating body and a regulatory framework. This has resulted in independent, asynchronous delivery of insecticide for marginal gain.

Fruit and vegetable production regions are often characterised by long growing seasons, diverse commodities, and polyphagous pests (such as tephritid fruit flies) that take advantage of these spatially and temporally heterogeneous landscapes that span the urban and rural habitats (Schwarzmueller et al. 2019). Often urban landscapes provide permanent low-density resources, such as a variety of backyard trees as potential pest hosts. Even though host plant density and diversity is much lower than in a commercial orchard setting, these urban environments can significantly contribute to high and persistent pest populations because populations are often uncontrolled and backyard hosts can fill the seasonal gaps by providing continuity of resources for pests (Schwarzmueller et al. 2019).

Beyond the biophysical challenges of AW-IPM in diverse landscapes are the psychosocial barriers for those who are directly (growers) and indirectly (regional towns people) affected by the pests. One of the greatest barriers to acceptance of communities participating in area-wide management of Queensland fruit fly was lack of social cooperation amongst growers such as insufficient care and responsibility about their role in the broader horticultural and social systems (Mankad et al. 2017). Increased transparency of the problems and issues for all actors, including better insights into knowing when pests show up, and where pests are located, may enable coordinated control and validation as to whether the AW-IPM actions are effectively suppressing the target population.

5. CONCLUSIONS

The inability to know where and when pests arrive, as well as their population dynamics in space and time, the inability to communicate this information in a timely manner and delivering coordinated pest control are barriers to AW-IPM, and in turn, potential barriers to efficient and sustainable pest management.

Increasingly technologies that free complex computation from desktop computing to in-field mobile devices are central to the development of in-field data collection, and automated insect detection and monitoring. Each of these will enable information communication among stakeholders, and the area-wide coordination of pest control.

Engineering solutions to overcome these barriers are many and varied. For government biosecurity, detecting rare events of pest incursions or escapees can minimize the size and duration of the management response, the cost, and the amount of time that markets are closed. For pest management of endemic species, automated insect detection solutions that are fast and easy to deploy, that provide real-time data flow, and that are cost-effective, can have a significant impact by improving estimates of pest numbers, pest locations and effectiveness of integrated control.

Even if it is early days, current in-field data collection and insect automation technology is already providing better information for more informed pest management decisions, improved workflow, and has the potential to reduce the harm from exotic pest incursions.

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PROSPECTS FOR REMOTELY PILOTED AIRCRAFT SYSTEMS IN AREA-WIDE INTEGRATED PEST MANAGEMENT PROGRAMMES

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SUMMARY

The scientific and technical advances achieved in recent years in the technology for *remotely piloted aircraft systems* (RPAS) and the approval of new regulatory frameworks in several countries have allowed the commercial expansion and use of these flying robots for different civil applications, including agriculture. The present review discusses the opportunities for the use of the RPAS in area-wide integrated pest management (AW-IPM) programmes within the current technical and legal constraints. These include targeted insecticide applications of hotspots in fruit fly and mosquito pest management programmes, aerial release of sterile males in mosquito and tsetse control programmes, and aerial release of parasitoids. The advantages of the RPAS technology - accuracy, increased safety and cost-efficiency for small and medium scale operations - are counterbalanced by its limitations at the technical level - reduced payload and flight duration - as well as at the regulatory level - mandatory special operational permits from regulatory agencies for operations beyond the visual line of sight.

Key Words: RPAS, unmanned aircraft, UAS, drones, regulation, sterile insects, parasitoids, pest control

1. INTRODUCTION

Area-wide integrated pest management (AW-IPM) programmes aim at controlling a given pest at a geographic scale, targeting the whole pest population (Hendrichs et al. 2007). By definition, there is an intrinsic spatial dimension in all area-wide programmes. The best way to operate at this geographic/spatial dimension is by combining geographic information systems (GIS) technology with aerial intervention tools (IAEA 2006). Until now, aircrafts have been widely used in AW-IPM implementation, and their deployment has major comparative advantages for those programmes operating over large areas of difficult topography and that lack road networks (Vreysen et al. 2007; Dyck et al. 2021). Although the technology of *remotely piloted aircraft systems* (RPAS) continues

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improving for many applications, it is already technically sufficiently mature to allow carrying out many of the activities of an AW-IPM operational programme by air. The term RPAS is the official International Civil Aviation Organization (ICAO) term for such aircraft, whereas terms such as *unmanned aircraft systems* (UAS) are being less used in view that regulations prescribe that all aircraft need to be piloted, even when the pilot is not on board (FAA 2017; CASA 2018). As the term *drone*, common in francophone countries, is increasingly identified with military applications, it is not used here.

The main current applications of RPAS in agriculture are air-borne scouting of field crops and of ranging livestock through remote sensing, as well as precision delivery systems and aerial spraying with low or ultra-low volumes in some crops. The advantages of the RPAS technology are mainly in the fields of safety, accuracy and cost efficiency. However, as it usually happens with technology involving a shift of paradigm in the way society deals with problems, the regulatory framework is not fully developed and has not evolved at the required speed. This has led some pioneers to develop applications with an *ad hoc* certificate of authorization (COA) or, in some cases, to operate at the fringes of the legal regulation. The future of RPAS will therefore depend as much on decisions made by regulators as it does on technological advances, and for example France's relatively permissive regulation has put it at the forefront of the agricultural use of unmanned aircrafts (The Economist 2017). In recent months, however, more appropriate regulatory frameworks have been adopted in different countries, clarifying the requirements for the operation of UAV and opening the field for commercial applications of RPAS, while at the same time, driving technology adaptation to the new regulations.

2. THE TECHNOLOGY

2.1. Elements of a Remotely Piloted Aircraft System (RPAS)

A RPAS is made of three main parts: the remote-control station, the datalink and the remotely piloted aircraft or (RPA) unmanned aerial vehicle (UAV) (Fig. 1). The control station contains all the elements that permit operators to manage the UAV flight. This includes the software that interacts with the aircraft via datalink, but also the computer (which can be just a tablet) and the joystick that controls the RPA in manual mode.

The datalink refers to the main elements that allow communications between the control station and the aircraft. The core element is the radio, which must use a frequency authorized by national authorities (for civil aviation purposes, usually 2.4 GHz or 900 MHz for remote control, along with 5.8 GHz for video and audio links) and can include helpful methods to avoid interferences, such as the "Frequency Hopping Spread Spectrum" (FHSS) technique. Communications can be digital or analogue: while the first provides higher quality, especially for video transmission, the range of the second one is higher for low-cost systems. The use of antennas and amplifiers should be considered too, especially when the payload and power capacity of the aircraft is too limited. For very long-range communications, it is highly recommended to use a tracking antenna: a directional antenna with embedded control actuators and installed encoders that automatically points to the aircraft with high precision, maximizing the communications range.

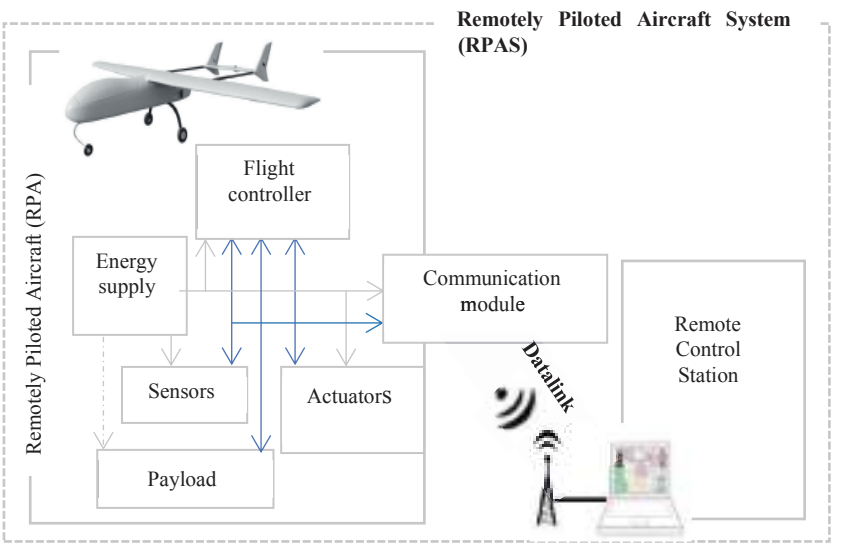

Figure 1. Main components of Remotely Piloted Aircraft Systems (RPAS) (modified after Wikipedia 2019).

Different options of unmanned vehicles are available to fulfil the required operation in the most efficient way: from fixed-wing aircrafts to multirotors, helicopters, blimps, parafoils or hybrid Vertical Take-Off and Landing (VTOL) platforms, among many others (Fig. 2).

There is also a wide variety of engines available for these vehicles, from electrical to fuel powered engines. Although not commonly used yet, remarkable progress has been achieved in the development of hydrogen fuel cell powered engines, which are expected to extend the flight endurance. The choice of the engine will depend on the autonomy requirements but also on the size and weight of the aircraft; sometimes altitude can be a limiting condition due to lower air density at high elevations above sea level, requiring for instance the use of electrical fuel injection (EFI) engines for higher efficiency. The payload capacity and flight duration are also key elements for the majority of operations and must be carefully balanced when selecting the RPA for any specific task.

Commonly related to the three mentioned elements of RPAS, autopilots are key in any unmanned operation, especially when the aircraft should perform autonomous missions without the constant control of operators. The most sophisticated autopilots have embed a suite of sensors and processors together with Line of Sight (LOS) and Beyond Radio Line of Sight (BRLOS) Machine to Machine (M2M) datalink radios, all with reduced size and weight to minimise the use of the payload capacity of the aircraft.

These autopilots usually permit the connection with external peripherals such as transponders and radars, increasing the capabilities of the platform during fully unmanned missions through the provision, for instance, of reliable and autonomous Detect & Avoid (DAA) tools. These refer to the technology that provides to unmanned vehicles the capability to detect obstacles on their route (such as other aircrafts) and to immediately find a way to prevent collision.

When they are aimed at professional purposes, autopilot systems must be developed according to international standards in order to be compliant with national regulations for the professional operation of RPAS.

Figure 2. Types of unmanned vehicles: (A) Fixed-wing; (B) Multirotor; (C) Helicopter; (D) Hybrid Vertical Take-Off and Landing (VTol); (E) Blimp; (F) Parafoil; (G) Unmanned surface vehicle (USV); (H) Optionally piloted vehicle (OPV); and (I) Unmanned ground vehicle (UGV) (credit Embention).

2.2. Safety Considerations

In addition to the design and development of the RPAS according to aerial international standards, redundant systems greatly reduce the risk of failure. Redundancy usually is focused on the autopilot (normally with triple redundancy). Triple redundancy for autopilots refers to the inclusion of three autopilot units in the same flight controller. These three autopilots operate as if they were a single unit dealing with the control of the unmanned platform. A dissimilar arbiter (a piece of electronics included in the redundant autopilot) decides which of those three autopilots is in command attending to the efficiency and coherence of their orders. Therefore, in case any autopilot unit fails, there will be still two units capable to deal with the whole operation. For critical operations performed by large aircraft, such as originally manned helicopters that were adapted into unmanned platforms, redundancy is commonly extended to actuators, as long as the platform can tolerate the increase in weight caused by the redundant actuators and the budget of the project can deal with their price.

Autopilot systems have several configurable safety routines that can be triggered under specific situations. Some of these safety routines include landing if the battery/fuel is below a defined threshold of its capacity, go back to the take-off point if the datalink

is lost, and reduce altitude if the wind speed is higher than a predefined value. Also, configurations aimed to end the flight in the safest possible way can be configured into the Flight Termination System (FTS): an arbiter microprocessor within the autopilot that activates the flight termination in case of failure of the main microprocessor or any of the motors (e.g. by releasing a parachute).

The most sophisticated autopilot systems also include sensors such as radars, lidars and/or transponders that permit the use of DAA technologies. An officially certified DAA system is currently mandatory to operate beyond visual line of sight in most of the countries, but due to the novelty and impact of this technology in the industry, currently the certification process of this kind of system consists of a series of negotiations with national authorities who normally request clear and irrefutable proof about the reliability of the system.

3. CURRENT APPLICATIONS OF THE RPAS TECHNOLOGY IN AGRICULTURE

Precision agriculture, construction and public safety are the most promising commercial and civil aviation markets for RPAS (The Economist 2017). RPAS are used in agriculture for low altitude remote sensing (LARS) of crop conditions with multi-spectral and hyperspectral cameras. High spatial resolution information on crop hydric stress (Berni et al. 2009), crop vegetation index (Primicerio et al. 2012), weed detection (Hardin et al. 2007) and yield mapping (Swain et al. 2010) can be obtained with the proper data acquisition and analysis.

When compared to satellite remote sensing, RPAS-based remote sensing has a higher spatial and temporal resolution, lower costs and is not affected by cloud coverage among other advantages (Nansen and Elliott 2016). However, the calibration of the sensors under field conditions and post-processing of the acquired information remain complex and require high level of expertise.

After analysing the data collected by RPAS, farmers can tailor their use of irrigation, pesticides, herbicides and fertilizers, applying by ground variable rate technologies and following the principles of precision-agriculture (Zhang and Kovacs 2012).

At the research level, remote sensing with RPAS has also been used to detect host plant responses to insect infestations, like the analysis of the variation in the vegetation index (Normalized Difference Vegetation Index or NDVI) in wheat fields caused by an attack of fall armyworm *Spodoptera frugiperda* J.E. Smith (Zhang et al. 2014).

RPAS are also used for low and ultra-low volume aerial spraying of insecticides and herbicides. In Japan, one of the world-leading manufacturers of agriculture RPAS (Yamaha Motor Company) estimates that about one million ha of rice, around 35% of the Japanese rice fields, are currently sprayed with their unmanned helicopters (Yamaha 2017).

In China, several commercial companies offer off-the-shelf commercial RPAS models for crop spraying with variable tank capacities and spraying methods, from thermal fogging to electrostatic spraying (DJI 2017; Joyance 2017). The mission planning and operation of these spraying RPAS is relatively simple thanks to dedicated remote controllers and software. Variable rates of pesticides, fertilizers and herbicides can be applied.

4. REGULATORY FRAMEWORK

4.1. Classification of Remotely Piloted Aircrafts and Operations

Although different legal frameworks exist in each country/region, most of the existing regulations classify the RPAs in relation to their weight (ICAO 2015; and other regulations listed in Table 1):

1. Micro-RPA if less than 2 kg of maximum take-off mass (MTOM)
2. Small RPA between 2 and 25 kg of MTOM and
3. Large RPA between 25 and 150 kg of MTOM.

They also classify them in relation to the type of operation:

1. Within the visual line of sight (VLOS) when the distance to the operator is less than 500 m with good visibility conditions and flight elevation below 120 m
2. Beyond visual line of sight (BVLOS) and flight elevation below 150m over the ground (also called Very Low Level or VLL operations)
3. Beyond visual line of sight (BVLOS) and flight elevation above 150 m over the ground; and
4. A fourth and intermediate class, which is the Extended Visual Line of Sight (EVLOS) for those operations where the main pilot has no visual contact with the RPA, but other pilots/observers, in communication with the main one, are capable to see it.

4.2. Different Regulations in Each Country

The regulatory framework for the operation of RPAs is not fully developed in all the countries and not fully harmonized across the countries and regions. However, some common ground among the regulations of different countries can be found:

1. Regarding the weight of the RPAs, those over 2 kg MTOM must be operated by certified operators; those over 25 kg MTOM must be registered and follow either a risk-based approach or obtain a type certificate.
2. Regarding the type of operation, RPAs should be operated within the VLOS. Operations BVLOS are only allowed under especial conditions (when the aircraft is under 2 kg, when operating in special areas of the airspace, or when using DAA technology or UAS Traffic Management (UTM)/U-Space systems (set of new services relying on a high level of digitalisation and automation of functions and specific procedures designed to support safe, efficient and secure access to airspace for large numbers of drones) (SESAR-JU 2019).
3. Operations over restricted areas (e.g. populated areas) must be performed by platforms with a certificate of airworthiness and also with permission from public authorities.
4. RPAS operators and RPAs above 250 g must be registered.
5. Most, if not all, RPAS operations must be insured.

Some examples of the relevant regulations for RPAS operators in different countries can be found in Table 1. Due to the early stage of the unmanned industry and regulations, waivers by the competent national authority against these regulations are possible.

Table 1. Regulatory framework for the use of remotely piloted aerial systems (RPAS) in different countries/regions

Country / Region	Organism	Document	Release Date
Australia	Civil Aviation Safety Authority (CASA 2018)	- General information - Regulation: Direction-operation of certain unmanned aircraft	2017-10
European Union	European Aviation Safety Agency (EASA 2017)	- General information - Opinion No 01/2018 Introduction of a regulatory framework for the operation of unmanned aircraft systems in the 'open' and 'specific' categories	2017-05
Japan	Civil Aviation Bureau (CAB 2016)	- General information	2015-10
Mexico	Dirección General de Aeronáutica Civil (DGAC 2017)	- General Information - Regulation: CO AV-23/10 R4 que establece requerimientos para operar un sistema de aeronave pilotada a distancia (RPAS)	2017-07
Spain	Agencia Estatal de Seguridad Aérea (AESA 2018)	- General information - Regulation: Utilización de RPAS	2017-12
USA	Federal Aviation Administration (FAA 2017)	- General information - JO 7200.23: Unmanned Aircraft Systems (UAS)	2016-10

4.3. Certificate of Airworthiness

The certificate of airworthiness is issued by the competent agency in charge of aviation safety in each country. Considering that it depends on national rules, this certificate may be mandatory if the maximum take-off mass (MTOM) of the remotely piloted aircraft is over 25 kg, but there are other situations where the certificate may be requested such as performing operations over populated areas or if the operation will take place BVLOS.

The request for a certificate needs to include every element of the RPAS, which includes a study about the risk of operating the system, the results of the tests made to the autopilot (hardware and software) and the structure of the aircraft and its resistance to harsh environments, such as extreme temperatures, humidity or dust conditions.

4.4. Pilots Qualifications

Regulations in most countries require RPAS pilots to be of legal age and their medical condition certified by an official aeronautical centre. In addition, the pilot must have the theoretical knowledge (an official license is required, such as an ultralight flight pilot license) and the practical knowledge on the specific RPAS (obtained from the manufacturer of the RPAS or by an organization delegated by the manufacturer) (ICAO 2015; and other regulations listed in Table 1).

5. ADVANTAGES AND OPPORTUNITIES FOR RPAS IN AW-IPM WITH THE CURRENT LEGAL REGULATIONS

One of the main advantages of the technology is in the field of safety, avoiding the risks for pilots inherent to any operation with manned aircrafts in agriculture. In case of accident, the severity of harm is minimised thanks to the absence of an onboard pilot and the lower kinetic energy of the aircraft.

Accuracy of the operations is very high (the same as in regular aircrafts), since the mission events are preconfigured based on geographic information and triggered automatically during the flight by the GPS information, avoiding human errors during operation. The parameters of the operation, such as the dose to be applied per surface unit during an aerial spraying operation or insect release, are controlled directly by the microprocessors that will adjust the actuators to obtain a constant value regardless of the variations of the flight speed (DJI 2017).

Cost-efficiency is also a major advantage of the RPAS technology thanks to the relatively low investment, maintenance and operational costs. However, cost-efficiency varies depending on the scale of the operation and detailed analysis should be conducted on a case by case basis.

Within the current legal framework, some of the activities that are part of the AW-IPM programmes for the control of different pest insects can be carried out using RPAS (Table 2). Some of the operations, such as the suppression of fruit fly hotspots, do not require a specific authorization from the civil aviation authority (CAA). Others, like those who require operation BVLOS, require a special operation permit by the relevant CAA based on a specific operations risk assessment (JARUS 2017).

5.1. Suppression of Fruit Fly Hotspots and Mosquito Larval Breeding Sites

One common activity in AW-IPM programmes against fruit flies is the suppression of hotspots by bait-spraying. Hotspots are detected and delimited by weekly trapping surveys and later normally treated by ground with bait sprays. For example, the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) control programme in Valencia (Spain) uses a fleet of all-terrain vehicles (ATV) equipped with ground sprayers for this purpose (Argilés and Tejedo 2007) (Fig. 3). However, the low volume of insecticide required by bait sprays compared to cover sprays and the limited size of the area to be treated – the size of hotspots is usually some tens of ha – make this task technically suitable for aerial application by RPAS.

Currently, off-the-shelf RPAS for spraying have been developed by several commercial companies with specific software and remote controller for this task. From the regulation point of view, the operation in VLOS over commercial crops outside restricted areas and with a maximum take-off mass below 25 kg exempt this application in most countries of the requirement for a specific permit or certificate of airworthiness from the national civil aviation authorities. The area covered per day can range between 25 to 50 ha depending on the insecticide application specifications, which is several times higher than can be achieved by ground spraying.

Table 2. Summary of technical parameters for different RPAS applications

Activity	Weight of adults		Release density		Biomass of insects per surface		Area to be covered per flight		Swath widths	
	min	max	min	max	min	max	min	max	min	max
Release of sterile *Aedes* mosquitoes	0.9 mg	1.1 mg	1000 males/ha	2000 males/ha	90 g/km^2	220 g/km^2	40 ha	60 ha	75 m	100 m
Release of sterile tsetse flies	20 mg	30 mg	0.5 males/ha	1.0 males/ha	1 g/km^2	3 g/km^2	3000 ha	8000 ha	300 m	400 m
Fruit fly hotspot bait spraying	-	-	-	-	-	-	3 ha	6 ha	8 m	10 m

Activity	Flight distance (without ferry)		Speed during operation	Flight time (without ferry)		Payload	Type of aircraft	Type of operation	Specific authorization by relevant CAA
	min	max		min	max				
Release of sterile *Aedes* mosquitoes	5.3 km	6.0 km	10 m/s	9 min	10 min	300 g	rotationary; micro	VLOS; urban area	mandatory
Release of sterile tsetse flies	100 km	200 km	20 m/s	83 min	167 min	900 g	fixed-wing/ helicopter; medium	BVLOS; rural area	mandatory
Fruit fly hotspot bait spraying	3.8 km	6 km	8 m/s	8 min	13 min	10 kg	rotationary; medium	VLOS; rural area	exempted

Similarly, mosquito larvicides can be applied aerially to larval breeding areas, replacing the current ground and helicopter treatments, as demonstrated in the ongoing tests by L'Entente Interdépartementale pour la Démoustication du Littoral Méditerranéen in France (EID Méditerranée 2018).

Figure 3. (A) Delimiting the extent of hot spots with GIS-based field surveys of monitoring traps. (B) Green tracks showing the bait spray ground treatment done in different citrus orchards; only orchards with a ripening variety are treated. (C) ATV applying a bait spray treatment by ground. (D) Spraying RPAS with a 10 litre tank.

5.2. Release of Sterile Aedes Mosquitoes in SIT Programmes

The weight of adult *Aedes spp.* mosquitoes is low (ca. 1 mg). They also have a low dispersal behaviour, which compared to other pests, makes it possible to apply area-wide control programmes over relatively small urban areas. These two conditions make the aerial release of sterile mosquito males technically feasible using RPAS.

Flying over the urban or peri-urban scenario typical of an *Aedes* spp. mosquito control programmes, however, requires a mandatory permit and certificate of airworthiness for the specific operation by the local aerial safety agency. To facilitate obtaining these, the use of micro-RPAS, i.e. under 2 kg of maximum take-off mass, is advised.

An aerial platform of less than 1 kg, including the payload with the release machine and an insect cargo of around 75 000 sterile males, can suffice to release mosquitoes in VLOS over 50 ha with a flight duration of less than ten minutes per flight and a flight elevation under 100 m.

5.3. Release of Sterile Tsetse Flies in SIT Programmes

Tsetse fly SIT programmes are applied at a scale of very large areas, usually covering several hundred or thousand km^2 of rural landscape (Feldmann et al. 2021). The required release density of sterile males is only between 50 to 100 sterile males per km^2 and per week (equivalent to 1 to 3 g of insects per km^2 and per week, depending on the tsetse species). This is a very low release rate when compared to other pest species, namely fruit flies (Hendrichs et al. 2021). Release swaths of 300-400 m are common in view that tsetse flies disperse sufficiently between flight lines.

To conduct the aerial releases under these conditions, a remotely piloted aircraft with a flying endurance of at least 100 km and a payload of approximately 1 kg for the chilled insect release machine and the sterile insect cargo is needed (for an example see Fig. 4). Several fixed-wing and helicopter models currently available on the market with a MTOM below 25 kg meet these technical requirements. The release operation will necessarily be BVLOS, and thus will require specific authorization by the local aerial safety agency. In addition, the take-off and landing point will need to be as close as possible or within the release area to save ferry flying time, which may require a field insectary.

Figure 4. Unmanned aircraft developed for the Southern Tsetse Eradication Project in Ethiopia. The pods under the wings carry the sterile tsetse males in chilled conditions.

5.4. Release of Parasitoids in AW-IPM Programmes

Several AW-IPM programmes release parasitoids to control different pests, such as the aerial releases of chilled *Diachasmimorpha longicaudata* (Ashmead) parasitoids mass-produced by the Moscafrut programme in Mexico to control *Anastrepha ludens* (Loew) and *An. obliqua* (Macquart) fruit flies (Montoya et al. 2007); or ground releases in Costa Rica of *Spalangia endius* Walker to control the cattle pest *Stomoxys calcitrans* (L.) by Costa Rica's National Institute of Agricultural Technology, Innovation and Transfer (INTA) (Solorzano-Arroyo et al. 2017). The release of these tiny wasps can be carried out with RPAs under 25 kg, although to be more cost-effective, the operation needs to be BVLOS, and therefore requires the specific authorization by the local aerial safety agency based on a specific operation risk assessment (JARUS 2017).

In Mexico, aerial releases of *Tamarixia radiata* Waterston, the parasitoid of *Diaphorina citri* Kuwayama, the vector of Huanglongbing, are being conducted with an

RPAS by the company Mubarqui within the area-wide programme of the Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria (SENASICA) that manages the citrus greening programme in Mexico (García-Ávila et al., this volume).

5.5. Release of Sterile Fruit Flies in SIT Programmes

The release of sterile fruit flies in SIT operational programmes is carried out with small aircrafts covering tens of thousands of ha per flight and with a cargo above 100 kg of biomass (FAO/IAEA 2017). RPAS doesn't seem suited for this job, since this would require large RPAs with a complex certification process.

Small pilot projects could possibly benefit of RPAS for aerial releases provided they apply a shift of strategy for the aerial releases, e.g. several short flights with a low load of sterile insects from a mobile base within the release area.

5.6. Strategy and Logistics for the Releases of Sterile Insects

As described, RPAS can potentially be used in some AW-IPM programmes to release sterile insects. To do so, some adaptations to the current release procedures are needed to compensate for the lower payload and endurance capacities of the RPA aircraft. Currently, large numbers of sterile insects are released in SIT operational programmes by manned aircrafts in a single flight covering large areas (for example, tens of millions of sterile insects over hundreds of km^2 in the case of fruit fly programmes).

If RPAS are to be used for the aerial release of sterile insects, multiple and shorter flights are needed for the same purpose. Currently, the manned aircrafts take-off from a runway located close to the sterile fly emergence and release facility. Release by RPAS will require that the sterile insects be transported by ground to the take-off point within the release area, or at least in the very close vicinity. This will require the use of a mobile take-off station from which the RPAS is operated and where sterile insects are kept under chilled conditions waiting for their turn to be released.

5.7. Release Machines

Apart from the mandatory requirements of using light materials, the release machines also require a chilling system capable of maintaining the temperature between 4°C and 8°C in the insect holding container, depending on the insect species, during the flight. Passive chilling systems like those based on the use of phase-change materials (PCM) can provide such conditions at a low payload cost. Relative humidity must be kept under the dew point to avoid condensation on the surface of the insect bodies. Some insects like mosquitoes or some species of parasitoid wasps have fragile legs and antennae and require higher holding temperatures and special equipment to avoid mechanical damage by compaction and vibrations during the flight and by friction while going through the release mechanism (Montoya et al. 2012).

Accurate releases with variable release rates can be continuous, along a flight path or discrete, focused on individual waypoints with predefined coordinates.

6. CONCLUSIONS

The RPAS technology is widely and increasingly used in agriculture for remote sensing and pesticide spraying of crops, mainly at the level of individual larger farms. However, RPAS are still not routinely used in operational AW-IPM programmes, such as fruit fly SIT programmes, because the scale of these programmes is usually too large for the current technical and regulatory limitations of RPAS.

Nevertheless, as discussed in this review, the legal regulations recently approved in several countries have opened the possibility to use the existing commercial RPAS technology for some of the field activities of AW-IPM programmes. This is the case of the chemical treatment of hotspots in fruit flies or mosquito control programmes, providing high application accuracy.

Some other applications, such as the release of sterile males of tsetse or mosquitoes, respectively over large or urban areas, require a specific risk assessment prior to the authorization by the relevant civil aviation authority in the country. In this regard, the development of the novel DAA technology and UTM/U-Space systems, and their official certification, will facilitate the authorization of applications and operations beyond the pilot VLOS, as commonly needed in area-wide programmes.

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Enterobacter: ONE BACTERIUM - MULTIPLE FUNCTIONS

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SUMMARY

Insects represent the most successful taxon of eukaryotic life, being able to colonize almost all environments. Microbial symbiomes associated with insects, impact important physiologies, and influence nutritional and immune system status, and ultimately, fitness. A variety of bacterial phyla are commonly present in insect guts, including Gammaproteobacteria, Alphaproteobacteria, Betaproteobacteria, Bacteroidetes, Firmicutes, Clostridia, Spirochetes, Verrucomicrobia, Actinobacteria, and others. Among them, the genus *Enterobacter* has been recognized as a dominant inhabitant of the gut for several important insect species, indicating an essential functional role for this taxon. Here, we review the known *Enterobacter* functional diversity among insects with respect to insect development, host exploitation, reproduction, and interactions with other organisms, in an attempt to provide an overview of the traits that have resulted in their evolutionary success. Many strains of *Enterobacter* species are not simply insect commensals but confer beneficial traits to their hosts that primarily fall into two categories: provision and degradation of nutrients and protection from pathogens.

Key Words: Microbial symbiomes, endosymbiotic bacteria, bacterial functional diversity, mutualisms, commensalisms, pathogenic relationships, nutrient degradation, nutrient provision, pathogen protection

1. INTRODUCTION

Microorganisms are well recognized as essential members of the biosphere. Over billions of years they have evolved into every conceivable niche on the planet. Microorganisms reshaped the oceans and atmosphere and gave rise to conditions conducive to the development of multicellular organisms (Gibbons and Gilbert 2015). Microbial diversity and distribution were mostly inaccessible and underestimated before the advent of molecular fingerprinting and high-throughput sequencing technologies, which now allow us to circumvent culture-based approaches (Fierer and Lennon 2011; Whitman et al. 1998).

J. Hendrichs, R. Pereira and M. J. B. Vreysen (eds.), Area-Wide Integrated Pest Management: Development and Field Application, pp. 917–945. CRC Press, Boca Raton, Florida, USA.

Bacteria and archaea estimates continue to rise within an increasing number of environments sequenced using advanced molecular techniques, with the number of existing microbial species predicted to reach millions (Brown et al. 2015; Williams et al. 2017; Zaremba-Niedzwiedzka et al. 2017).

The term symbiosis originates from the Greek word "symbioun" meaning "to live together"; it was defined by Anton de Bary in 1879 as "the living together of two dissimilar organisms, usually in intimate association, and usually to the benefit of at least one partner" (Bary 1879). Symbiotic bacteria are omnipresent in all types of ecosystems, having a significant impact on eukaryotic evolution and diversity (Martin and Schwab 2012; McFall-Ngai 2007; Ruby et al. 2004). Although considerable attention has been given to pathogenic bacteria, pathogens are a minute minority of animal symbionts.

Microbial symbioses are generally categorized as parasitism, commensalism, or mutualism, though some relationships may wander across these defined boundaries depending on evolutionary processes, changes in environmental conditions and/or health state of the host/symbionts (Webster 2014). Host development, defence, nutrient assimilation and disease, in humans and other animals, is influenced by microbial mutualisms, commensalisms and pathogenic relationships (Eren et al. 2015). The development of next-generation DNA sequencing platforms has allowed an in-depth understanding of the composition of the microbial populations inhabiting different hosts and symbiosis research has focused on associations that (i) have economic importance, (ii) have implications for human health, or (iii) offer ecologically fascinating insights (Webster 2014).

It is estimated that the majority of members of the largest class of invertebrates, i.e. Insecta, are involved in some type of symbiosis, with most of these relations being shared with bacteria. Microorganisms can colonize the insect exoskeleton, the gut and haemocoel, and are present within some insect cells. The microbiota may account for 1–10% of the insect biomass, implying that the insect, as well as any other higher organism, can be regarded as a multi-organismal entity (Douglas 2015). Bacterial symbionts are equally prevalent in mammals and insects; however, the bacterial diversity in insect digestive tracts is generally low and rarely exceeds a few tens of species (Colman et al. 2012). Several immunological, physiological and morphological hypotheses have been proposed to explain that fact (Broderick and Lemaitre 2012; Engel and Moran 2013). The lack of adaptive immune function in invertebrates might partly explain this low diversity (McFall-Ngai 2007).

2. INSECT SYMBIONTS

Insect-associated microorganisms cover an immense range of functions and are known to upgrade nutrient-poor diets; aid digestion of recalcitrant food components; protect from predators, parasites, and pathogens; contribute to inter- and intraspecific communication; affect efficiency of disease vectors; and govern mating and reproductive systems (Engel and Moran 2013; Gil and Latorre 2019).

2.1. Obligate Mutualistic Symbionts

Insects that depend exclusively on nutritionally restricted diets such as plant sap, vertebrate blood and woody material, commonly possess obligate mutualistic symbionts that are involved in the provision of essential nutrients or the degradation of food materials (Engel and Moran 2013; Engel et al. 2016; Latorre and Manzano-Marín 2017). At least 10% of all insect species depend on obligate nutritional symbioses where bacteria are required to synthesize nutrients that are absent in the diets of their hosts (Wernegreen 2004). Aphids provide an example of such obligate endosymbiosis since all aphids harbour endosymbiotic bacteria (i.e. microorganisms that live inside host cells or tissues) of the genus *Buchnera* in specialized host cells called 'bacteriocytes' (Manzano-Marín et al. 2016). Other obligatory endosymbionts include *Wigglesworthia* in tsetse flies (Akman et al. 2002), *Blochmannia* in carpenter ants (Williams and Wernegreen 2010), *Baumannia* in sharpshooters (Wu et al. 2006), *Carsonella* in psyllids (Nakabachi et al. 2006) and *Tremblaya* in mealybugs (Szabó et al. 2017). In a broader definition in the same category, we can include *Candidatus* Erwinia dacicola in the olive fruit fly *Bactrocera oleae* (Capuzzo et al. 2005; Ben-Yosef et al. 2014). These intracellular mutualists commonly have the following biological features: (a) they are located inside bacteriocytes, (b) are essential for fitness, (c) are transmitted maternally, and (d) display strict host-symbiont co-evolutionary patterns (Bourtzis and Miller 2008).

*2.2. "Facultative" S*ymbiotic *Bacteria*

In addition to obligate symbionts, many insects harbour "facultative" symbiotic bacteria, which are not essential for either host survival or reproduction and are typically maintained with a patchy distribution in host populations. Some facultative symbionts, like *Wolbachia*, *Spiroplasma* and *Cardinium*, are famous as reproductive manipulators in insects, affecting host reproduction by inducing male-killing, feminization, parthenogenesis or cytoplasmic incompatibility (Zchori-Fein and Perlman 2004; Doudoumis et al. 2013, 2017; Mateos et al. 2018). It has been speculated that these reproductive manipulators are not only harmful agents but also accelerators of host speciation due to reproductive isolation between infected and uninfected hosts. At the same time, *Wolbachia* has been shown, under certain conditions, to protect their hosts from insect pathogenic viruses as well as to prevent the establishment and transmission of major human pathogens like dengue and chikungunya viruses and the malaria parasite *Plasmodium* (Schmidt et al. 2017; Tan et al. 2017; Ant et al. 2018).

2.3. Gut Microbiota

Research conducted mainly in the last ten years, has resulted in tremendous progress in the field of gut microbiota and their impact on host metabolism. In general, gut microorganisms provide several nutritional functions to their hosts, and in return, hosts provide symbiotic microorganisms with a stable, protected, and nutrient-rich environment (Kohl et al. 2014). All animals assemble and maintain a diverse but host-

specific gut microbial community. Aside from the ubiquitous gut microflora in vertebrates, numerous invertebrates harbour endosymbiotic microorganisms inside their body cavity (Feldhaar 2011). It is believed that the gut microbiota can be considered as a bacterial organ, which is integrated into the biological system of the host (Sandeva et al. 2018). Gut microorganisms are also considered endosymbionts (Moya et al. 2008), although they are localized extracellularly within the gut lumen and their persistence within the gut could range from transient visitors to permanent inhabitants (Feldhaar 2011). Gut microorganisms produce a diverse metabolite repertoire from the anaerobic fermentation of undigested dietary components that reach the colon, and from endogenous compounds that are generated by the microorganisms themselves and their hosts (Rooks and Garrett 2016). Increased availability of technologies that profile microbial communities revealed that gut microorganisms are distinct from those of other characterized habitats in the biosphere, which indicates that strong selective forces differentiated gut-dwelling bacteria regardless of their lineage (Ley et al. 2008).

The basic structure of the digestive tract is similar across insects, although they possess a diversity of modifications associated with adaptations to specialized niches and feeding habits, and many of these specializations have evolved for housing gut microorganisms in specific gut compartments (Engel and Moran 2013; Pereira and Berry 2017). A variety of bacterial phyla are commonly present in insect guts, including Gammaproteobacteria, Alphaproteobacteria, Betaproteobacteria, Bacteroidetes, Firmicutes, including *Lactobacillus* and *Bacillus* species, Clostridia, Spirochetes, Verrucomicrobia, Actinobacteria, and others (Colman et al. 2012). The contribution of microorganisms, particularly gut microorganisms, to insect function is highly relevant from several perspectives, relevant to medicine, agriculture, and ecology (Douglas 2015).

2.4. Unveiling the Black Box of Symbiotic Associations

Characterization, exploitation and management of the insect-microbial symbiotic associations can contribute significantly to the control of agricultural pests and disease vectors. Recent advances in "omics", such as metagenomics, metatranscriptomics, and metaproteomics, have gradually unveiled the black box of symbiotic systems. However, capturing the properties of insect microbiota has been challenging due to the high variability in composition between individuals and closely related species (Pernice et al. 2014). Several biological and ecological factors such as age, genetics and environment have been proposed to explain insect microbiota composition. However, diet is one of the main factors driving variation in the composition of the gut microbiota in vertebrates and invertebrates. In insects, host diet composition has been shown to shape the microbiota composition regardless of taxonomy and geography of the specimens (Colman et al. 2012; Chandler et al. 2011; Yun et al. 2014).

Food itself can be a vector of commensals, and different diets will provide microbial inoculates of different community compositions. Also, one major difference between the human and insect microbiota is that the majority of the insect bacteria seem to be aerobic and therefore capable of digesting food outside the insect. This

function may contribute to explaining why host diet is such a key driver of microbiota composition and explain why bacteria commonly associated with insects are very rare in diverse mammalian species and vice versa (Chandler et al. 2011; Pernice et al. 2014). Some insect-associated microorganisms are related to microbial taxa found in other animals, e.g. Enterobacteriaceae and other Gammaproteobacteria, Firmicutes and Bacteroidetes, but others are absent from any other animal and the external environment (e.g. many protists of the class Parabasalia are found exclusively within termites (Brune and Dietrich 2015)).

3. *ENTEROBACTER* A DOMINANT TAXON OF GUT MICROBIOTA WITH MULTIPLE ROLES

Members of the Enterobacteriaceae family are facultatively anaerobic, Gram-negative rods that are catalase-positive and oxidase-negative (Brenner et al. 1984). Currently, the family comprises 51 genera (Table 1) and 238 species. The number of species displays a wide divergence with a range of 1 to 22 per genus. Twenty-two genera contain only one species, while seven genera have more than ten species (Octavia and Lan 2014).

Table 1. The genera (51) of the family Enterobacteriaceae[*]

Arsenophonus (1991), *Biostraticola (2008), Brenneria (1999),* ***Buchnera (1991)****, Budvicia (1985), Buttiauxella (1982), Cedecea (1981),* ***Citrobacter (1932),*** *Cosenzaea (2011), Cronobacter (2008), Dickeya (2005), Edwardsiella (1965),* ***Enterobacter (1960),*** *Erwinia (1920),* ***Escherichia (1919),*** *Ewingella (1984), Gibbsiella (2011), Hafnia (1954),* ***Klebsiella (1885), Kluyvera (1981),*** *Leclercia (1987), Leminorella (1985), Lonsdalea (2012), Mangrovibacter (2010), Moellerella (1984),* ***Morganella (1943****), Obesumbacterium (1963),* ***Pantoea (1989), Pectobacterium (1945),*** *Phaseolibacter (2013), Photorhabdus (1993), Plesiomonas (1962), Pragia (1988),* ***Proteus (1885), Providencia (1962),*** *Rahnella (1981),* ***Raoultella (2001),*** *Saccharobacter (1990),* ***Salmonella (1990),*** *Samsonia (2001),* ***Serratia (1823), Shigella (1919),*** *Shimwellia (2010),* ***Sodalis (1999),*** *Tatumella (1982), Thorsellia (2006), Trabulsiella (1992),* ***Wigglesworthia (1995), Xenorhabdus (1979),*** *Yersinia (1944), and Yokenella (1985).*

[*]*The year the genus was proposed is listed in parentheses after the genus name; also, the genera of the family that have been characterized in insects are highlighted with bold text*

Numerous applications use members of Enterobacteriaceae, including biocontrol in agriculture, production of recombinant proteins and non-protein products, control of infectious diseases, anticancer agents, biowaste recycling, and bioremediation (Watanabe et al. 2000; Zhu et al. 2011; Zhang et al. 2012; Zhao et al. 2012).

The family Enterobacteriaceae is ubiquitous, and many species can exist as free-living in diverse ecological niches, both terrestrial and aquatic, and some are associated with animals, plants, or insects only (Octavia and Lan 2014). For convenience, the members are broadly categorized into three types: (1) those that can cause human infections or are primarily associated with human/animals and the environment, (2) those that are associated with plants or plant pathogens and the environment, and (3) those that are associated with insects or are endosymbionts.

The Enterobacteriaceae (Proteobacteria) are considered as one of the most dominant bacteria families associated with insects (Drew and Lloyd 1987; Behar et al. 2005; Jurkevitch 2011; Rizzi et al. 2013; Wang et al. 2014; Cambon et al. 2018). They gave rise to a variety of symbiotic forms, from the loosely associated commensals, often designated as secondary (S) symbionts, to obligate mutualists, called primary (P) symbionts (Husník et al. 2011). Many enterobacteria are also opportunistic pathogens.

The genus *Enterobacter* was first described by Hormaeche and Edwards (1960) and was named for the organisms' predominant natural habitat, the intestines of animals (from Greek *enteron*, meaning "intestine"). *Enterobacter* is a genus of common Gram-negative, facultatively anaerobic, rod-shaped, non-spore-forming bacteria of the family Enterobacteriaceae. The genus is polyphyletic based on the 16S rDNA sequence, with 14 lineages scattered across the 16S rDNA tree.

The taxonomy of *Enterobacter* has a complicated history, with several species transferred to and from this genus. The older species were assigned to the genus based on DNA-DNA hybridization values and phenotypic data; whereas the more recently described taxa rely on 16S rRNA gene- and *rpoB*-sequencing for genus allocation. It has been previously acknowledged that *Enterobacter* contains species which should be transferred to other genera. The polyphyletic nature of the genus makes it difficult to assign novel species to *Enterobacter* unless the strains cluster with the type species (*E. cloacae*) of the genus (Brady et al. 2013).

Members of the *Enterobacter* can be found in soil, water, sewage, vegetable and fruits, plants, terrestrial and aquatic environments. They can be isolated from the intestinal tracts of humans and other animals as commensals, but they are also significant human pathogens (Hoffmann and Roggenkamp 2003; Hoffmann et al. 2005; Davin-Regli and Pagès 2015).

Interestingly, *Enterobacter* spp. have been recognized as inhabitants of the gut of several insect species, suggesting that this genus may play various and vital roles (Vasanthakumar et al. 2006; Geiger et al. 2009; Jiang et al. 2012; Gujjar et al. 2017). In this respect, we reviewed the known *Enterobacter* diversity among insects in relation to the functional role in insect development, host exploitation, reproduction, and interactions with other organisms, in order to recognise the traits that have resulted in their evolutionary success. Many *Enterobacter* strains are not simply insect commensals but confer beneficial traits to their hosts that primarily fall into two categories: provision/degradation of nutrients and protection from pathogens.

3.1. Enterobacter *and Nutrient Bioavailability*

Many insects derive nutritional advantage from persistent associations with microorganisms that variously synthesize essential nutrients or digest and detoxify ingested food (Douglas 2009). These persistent relationships are symbioses, and the species of the genus *Enterobacter* often plays a leading role (Table 2). Endosymbiotic *Enterobacter* species add nutritional supplements to their host diet, most of the times in cooperation with other members of the insect gut microbiota, like *Klebsiella, Pectobacterium* or *Citrobacter*. In these interactions, the primary nutrient contributor may switch between the various bacterial species of these genera.

Table 2. Enterobacter *species provide nutritional supplements to their host diet*

Species	Strain Accession Number	Host		Function	Reference
E. agglomerans	Not available	*Coptotermes formosanus* Shiraki	Formosan subterranean termite	Nitrogen-fixing	Potrikus And Breznak 1977
E. sp.	Not available	*Coptotermes acinaciformis* (Frogatt), *Coptotermes lacteus* (Froggatt), *Cryptotennes primus* Hill, *Heterotermes ferox* (Froggatt), *Mastotennes darwiniensis* Froggatt, *Nasutitermes graveolus* (Hill), *Schedorhinotermes intermedius* (Brauer)	Termites	Nitrogen-fixing	Eutick et al. 1978
E. agglomerans	Not available	*Dendroctonus terebrans* (Olivier)	Bark beetles	Nitrogen-fixing	Bridges 1981
*E.*sp.		*D. frontalis* Zimmermann			
E. aerogenes		*Ips avulsus* (Eichhoff)			
E. cloacae	Not available	*Bactrocera tryoni (Froggatt)*	Queensland fruit fly	Nitrogen-fixing	Drew and Lloyd 1987; Murphy et al. 1994
E. sp.	Not available	*Anoplophora chinensis (Forster)*	Wood-boring beetle	Nitrogen-fixing	Rizzi et al. 2013
E. sp.	AB673459 AB673457	*Reticulitermes speratus (Kolbe),*	Termites	Recycling of uric acid nitrogen	Thong-On et al. 2012
	AB673458 AB673456	*Glyptotermes fuscus* (Oshima), *Cryptotermes domesticus* (Haviland)			
E. sp.	AY847157 AY847162	*Ceratitis capitata* (Wiedemann)	Mediterranean fruit fly	Nitrogen-fixing	Behar et al. 2005
E. hormaechei	KC759162	*Reticulitermes chinensis* (Snyder)	Termite	Lignin degradation	Zhou et al. 2017
E. sp.	JQ864378	*Reticulitermes chinensis* (Snyder)	Termite	Recycling of uric acid nitrogen	Fang et al. 2016
	JX291546				
E. aerogenes	Not available	*Mastotermes darwiniensis* Froggatt	Termite	Lignin degradation	Kuhnigk et al. 1994
E. cloacae	Not available	*Reticulitermes flavipes* (Kollar)			

E. aerogenes	Not available	*Mastotermes darwiniensis* Froggatt	Termites	Hemicellulose degradation	Schäfer et al. 1996
E. sakazaki	Not available	*Reticulitermes flavipes* (Kollar)			
E. aerogenes	Not available	*Coptotermes formosanus* Shiraki	Formosan subterra-nean termite	Cellulose/Hemi-cellulose degradation	Adams and Boopathy 2005
E. cloacae	Not available				
E. aerogenes	EU305608	*Coptotermes curvignathus* (Holmgren)	Termite	Lignocellulose degradation	Ramin et al. 2008
E. cloacae	EU305609				
E. cloacae	MF185378	*Rhynchophorus ferrugineus* (Olivier)	Red palm weevil	Cellulose degradation	Muhammad et al. 2017
E. sp.	JN167548	*Rhynchophorus ferrugineus* (Olivier)	Red palm weevil	Cellulose/Hemi-cellulose degradation	Butera et al. 2012
E. sp.	LN829595	Coleoptera	Wood-feeding beetle larvae	Lignocellulose degradation	Manfredi et al. 2015
E. sp.	HM771088	Cerambycidae, Elateridae,	Coleoptera larvae	Cellulose/Hemi-cellulose degradation	Rojas-Jiménez and Hernández 2015
	HM771065 HM771070 HM771068 HM771104 HM771106 HM771101 HM771061 HM771048 GU827537	Passalidae and Scarabaeidae			
E. sp.	HM235482 HM235485 HM235491 HM235497	*Sirex noctilio* Fabricius	Wood-feeding wasp	Cellulose degradation	Adams et al. 2011
E. asburiae, E. cloacae, E. sp.	KT957438-43	*Plutella xylostella* (Linnaeus)	Diamond-back moth	Cellulose/Hemi-cellulose degradation	Xia et al. 2017
E. sp.	Not available	*Diestrammena asynamora* (Adelung)	Greenhouse camel cricket	Cellulose/Hemi-cellulose degradation	Mathews et al. 2019
E. sp.	AB872360 -384	*Holotrichia parallela* (Motschulsky)	Dark black chafer beetle	Cellulose/Hemi-cellulose degradation	Sheng et al. 2015
E. cloacae	Not available	*Coptotermes curvignathus* (Holmgren)	Termite	Cellulose degradation	Toczyłowska-Mamińska et al. 2015
E. cloacae	KC512248	*Plutella xylostella* (Linnaeus)	Diamond-back moth	Trehalose hydrolysis	Adhav et al. 2019

E. cloacae	Not available	*Thermobia domestica* (Packard)	Firebrat	Water and nutrients	Woodbury and Gries 2013a,b
E. sp.	LJAN01000071 locus_taq:AC520_4801	*Bactrocera oleae* (Rossi)	Olive fruit fly	Amino acids and vitamins	Estes et al. 2018a
E. sp.	KR232642	*Ceratitis capitata* (Wiedemann)	Mediterranean fruit fly	Probiotic	Augustinos et al. 2015
E. cloacae, E. asburiae	KY810513 KY810514	*Ceratitis capitata* (Wiedemann)	Mediterranean fruit fly	Probiotic	Hamden et al. 2013
E.agglomerans	Not available	*Ceratitis capitata* (Wiedemann)	Mediterranean fruit fly	Probiotic	Niyazi et al. 2004
E. sp.	Not available	*Zeugodacus cucurbitae* (Coquillett)	Melon fly	Probiotic	Yao et al. 2017
E. sakazakii	DQ228421-22	*Stomoxys calcitrans* Linnaeus	Stable fly	Probiotic	Mramba et al. 2007
E. cloacae	Not available	*Plutella xylostella* (Linnaeus)	Diamond-back moth	Probiotic	Somerville et al. 2019

3.1.1. Nitrogen Fixation

A well-established contribution of *Enterobacter* species to nutrition is nitrogen fixation. Nitrogen fixation is a metabolic capability that is absent from the ancestral eukaryote and has been acquired by multiple eukaryotic groups through symbiosis. Many insects are known to live on low nitrogen diets, and microorganisms have been suggested to provide availability of these essential foodstuffs in various ways (Douglas 2009).

Potrikus and Breznak (1977) identified two *Enterobacter agglomerans* strains from the guts of Formosan subterranean termites (*Coptotermes formosanus*). Nitrogen fixation appears to play an essential role in termite biology by helping them overcome deficiencies related to their nitrogen-poor diet (wood). The process was found to be linked to the termite gut bacteria since antibiotic treatment eliminated the function (Breznak et al. 1973). Strains C-1 and C-2 were characterized with electron microscopy and numerous biochemical assays, including sugar fermentation tests. The nitrogen-fixing ability of the strains was verified with acetylene reduction tests, in both aerobic and anaerobic (100% N_2 or 100% Ar) growing conditions, using different sources of nitrogen (peptone, NH_4Cl, KNO_3). In media lacking peptone, NH_4Cl or KNO_3, nitrogen fixation by both strains occurred only under anaerobic conditions when 100% N_2 was provided. When peptone, NH_4Cl, or KNO_3 was provided in the media, fixation was taking place in both aerobic and anaerobic conditions (Potrikus and Breznak 1977).

Nitrogen fixation by *Enterobacter* species was also identified in the gut of termite species from Australia (in seven out of nine species tested) (Eutick et al. 1978). The *Enterobacter* strains could grow on nitrogen-free media under anaerobic conditions, but also under aerobic conditions in the presence of H_2SO_4 (Eutick et al. 1978).

Some years later, nitrogen-fixing *Enterobacter agglomerans* and *Enterobacter* spp. were isolated from various species of bark beetles, including *Dendroctonus terebrans*, *D. frontalis* and *Ips avulsus* (Bridges 1981). Even though large populations of nitrogen-fixing bacteria were found in the beetles, the *in-situ* assays in larvae did not reveal any acetylene reduction (Bridges 1981).

In the wood-boring beetle *Anoplophora chinensis*, enrichment studies of adult gut homogenates for nitrogen-fixing revealed the presence of four *Enterobacter* strains (Rizzi et al. 2013). The adult insects used in the enrichment cultures were isolated from *Acer saccharinum* L. and *Alnus* host trees (Rizzi et al. 2013).

Many microorganisms are valuable to the insect for their more comprehensive metabolic capabilities, including their capacity to utilize insect nitrogenous waste compounds (e.g. uric acid), synthesize ‘high value' nitrogenous compounds (e.g. essential amino acids) and fix nitrogen (Douglas 2009). Uric acid is another substance utilized as a nitrogen source in insects feeding on nitrogen-poor diets composed of plant material (Potrikus and Breznak 1981). In such a case, four uricolytic *Enterobacter* strains were isolated from the guts of three wood-feeding termite species (*Reticulitermes speratus*, *Glyptotermes fuscus* and *Cryptotermes domesticus*) (Thong-On et al. 2012). Bacteria with uricolytic activity were grown anaerobically on plates containing various concentrations of uric acid. Isolated strains, RsN-1, GfU-1, Cd20b and Cd15a, showed >98% identity with *Enterobacter amnigenus*, *E. aerogenes*, *E. asburiae* and *E. cowanii*, respectively (Thong-On et al. 2012).

Enterobacter cloacae strains isolated from field-collected, and laboratory-reared *Bactrocera tryoni* (Froggatt) fruit flies showed dinitrogenase activity and were able to fix atmospheric nitrogen (Drew and Lloyd 1987; Murphy et al. 1994). Diazotrophic *Enterobacter* strains were also isolated from gut tissue of field-collected Mediterranean fruit flies *Ceratitis capitata* (Behar et al. 2005; Augustinos et al. 2021). However, the most potent nitrogen-fixing effect was produced by *Klebsiella* and *Citrobacter* species. Nitrogen fixation activity was estimated by a variety of methods, including acetylene reduction assays in live flies and bacteria grown on nitrogen-deficient media, amplification of the nitrogenase iron protein gene (*nifH*) from gut extracts and isolated colonies, as well as *in situ* expression and amplification of the *nifH* gene (Behar et al. 2005).

3.1.2. Degradation of Plant Cell Wall Components

Various *Enterobacter* strains are also able to provide nutrients to insects through the degradation of plant cell wall components (lignin, cellulose, hemicellulose) (König et al. 2006). Lignocellulolytic *Enterobacter* species have been identified in plant or wood-feeding insects, like beetles, termites or wasps.

A ligninolytic *Enterobacter* strain was isolated from abdomens of adult *Reticulitermes chinensis* termites that use the plant cell wall polysaccharide in their diet (Zhou et al. 2017). Strain PY12, classified as *E. hormaechei* based on its 16S rRNA sequence, produced a lignin-modifying peroxidase (LiP), a key component in the lignin degradation pathway (Zhou et al. 2017; Janusz et al. 2017). The decolourization of six different dyes determined Lip activity by spectrophotometry. Since the study focused only on one of the ligninolytic enzymes in the pathway, it was not entirely possible to elucidate how strain PY12 contributes to lignin degradation and the possible synergies it develops (Zhou et al. 2017).

Seven *Enterobacter* strains with the ability to degrade lignin and related aromatic compounds were identified in hindguts of various laboratory-reared and field-collected species of termites (Kuhnigk et al. 1994). Degradation was observed under aerobic conditions, while in the absence of oxygen, only slight modifications of the side group of aromatic compounds occurred (Kuhnigk et al. 1994). The strains were characterized as *E. aerogenes* (Km3 and KAn8), *E. cloacae* (Rt5a, Rt5b and Rt5c) and *Enterobacter* sp. (Rt3a and Rt3b). The first two were isolated from *Mastotermes darwiniensis* and the remaining five from *Reticulitermes flavipes* termites. Among the seven strains, Km3 and KAn8 exhibited the most significant degradation potential, with the ability to degrade nine out of 13 substrates tested (Kuhnigk et al. 1994). Apart from degrading lignin, strain Km3 also showed hemicellulose degrading activity (Schäfer et al. 1996). Hemicellulose was also degraded by *Enterobacter sakazakii* strain RA2 that was isolated from hindguts of wild *R. flavipes* termites from France (Schäfer et al. 1996). Interestingly, strain RA2 exhibited all (four out of four) enzyme activities that were tested and were related to polysaccharide degradation. On the other hand, strain Km3 lacked 1,4-β-xylanase activity (Schäfer et al. 1996). In a particular environment, though, bacteria lacking members of the metabolic cascade could act synergistically with other strains present to fully degrade substances.

The Formosan subterranean termite, *C. formosanus* contained *E. aerogenes* and *E. cloacae* strains that effectively utilized xylose as a carbon source (Adams and Boopathy 2005). Two strains isolated from *Coptotermes curvignathus* termites from Malaysia and identified as *E. aerogenes* and *E. cloacae* were able to degrade carboxymethylcellulose (CMC) and cellobiose (Ramin et al. 2008). Other lignocellulolytic strains identified in higher and lower termites belonged to the species *E. aerogenes*, *E. agglomerans* and *E. cloacae* (König et al. 2006; Mannesmann and Piechowski 1989).

3.1.3. Degradation and Biosynthesis of Other Nutrients

In the diamondback moth *Plutella xylostella*, Adhav et al. (2019) characterized, structurally and biochemically, a trehalase from *Enterobacter cloacae* which assists in the hydrolysis of trehalose, a significant energy source in insect metabolism. Firebrat nymphs (*Thermobia domestica*) acquire water and nutrients by consuming *Enterobacter cloacae*, facilitating their growth and survival in the absence of food or water for up to 22 days (Woodbury and Gries 2013a,b).

Genome sequencing of *Enterobacter* sp. OLF (Table 3), isolated from wild California olive fruit flies, revealed genes related to the biosynthesis of amino acids, vitamins and co-factors, degradation pathways, nitrogen metabolism, as well as the production of energy and precursor metabolites (Estes et al. 2018a, 2018b).

This characterization could mean that the strain could potentially supply its host, in cooperation with other symbionts, with amino acids, vitamins or other nutritional compounds missing from the olive fruit diet (Estes et al. 2018a, 2018b).

Table 3. Available genome sequences of Enterobacter *symbionts from insects*

Accession number	*Enterobacter* Strain	Host	Status	Submission Date
PRJNA420145	*E. cancerogenus* CR-Eb1	*Galleria mellonella* (Linnaeus)	complete	2018
PRJNA288712	*E. sp. OLF*	*Bactrocera oleae* (Rossi)	complete	2018
PRJNA340971	*E. Larv1_ips*	*Ips typographus* (Linnaeus)	draft	2019
PRJNA390046	*E.* sp. 10-1	Cerambycidae	draft	2017
PRJNA364290	*E. JKS000234*	Formicidae	draft	2017
PRJNA364289	*E. JKS000233*	Formicidae	draft	2017
PRJNA179500	*E. hormaechei YT2*	*Tenebrio molitor* (Linnaeus)	draft	2012
PRJNA180991	*E. hormaechei* YT3	*Zophobas morio* Fabricius	draft	2013
PRJNA180988	*E. cancerogenus* YZ1	*Tenebrio molitor* (Linnaeus)	draft	2013
PRJNA169065	*E. sp.* Ag1	*Anopheles gambiae* Giles	draft	2012

3.1.4. Probiotic Effects of Enterobacter

The most recent contribution of *Enterobacter* strains to insect fitness and nutrition is their emerging role as probiotics in diets of mass-reared *C. capitata* and *Zeugodacus cucurbitae* flies. Such properties can be particularly useful in Sterile Insect Technique (SIT) applications where irradiation treatment takes a heavy toll on insect competitiveness if the dose administered is high. In this regard, strains that were provided as probiotics in diets greatly improved various fitness parameters of the flies including pupal weight, longevity, adult size, flight ability, and adult emergence (Niyazi et al. 2004; Ben Ami et al. 2009; Hamden et al. 2013; Yuval et al. 2013; Augustinos et al. 2015, 2021; Kyritsis et al. 2017; Yao et al. 2017; Cáceres et al. 2019). For example, the provision of *Enterobacter* sp. strain AA26 resulted in increased production of pupae and adults, as well as reduced rearing duration in various developmental stages (from egg to pupa, pupal stage and from egg to adult), particularly for male *C. capitata* flies (Augustinos et al. 2015). However, it did not affect pupal weight, sex ratio, male mating competitiveness, flight ability or life span under food and water deprivation (Augustinos et al. 2015; Kyritsis et al. 2017).

Hamden et al. (2013) on the other hand observed increased pupal weight, male sexual performance and survival rates under food deprivation when they enriched the larval diet of mass-reared *C. capitata* with *Enterobacter* sp. and other beneficial bacteria (*Klebsiella pneumoniae* and *Citrobacter freundii*). In terms of mating competitiveness, irradiated males reared on probiotics achieved more matings and transferred larger quantities of sperm to females. Finally, in addition to pupal weight, probiotics also resulted in increased body size for adult males (head width, abdomen and thorax length) (Hamden et al. 2013). Niyazi et al. observed a diet-dependent probiotic effect on the mate-calling activity, mating success, life expectancy, and survival of mass-reared male *C. capitata* flies (Niyazi et al. 2004). The probiotic effect of *E. agglomerans* and *K. pneumoniae* on each of these parameters differed among the four diets that were tested (two standard adult diets and two enhanced experimental formulations), ranging from significant to non-existent (Niyazi et al. 2004). Differences in the probiotic effect were also observed between the laboratory trials and the field-cage assay (Niyazi et al. 2004).

Similarly, Yao et al. (2017) tested gut-associated *Enterobacter* spp. as probiotics in the larval diet of irradiated laboratory-reared *Z. cucurbitae* flies. Both live and autoclaved bacteria were tested to distinguish between insect-bacteria interactions and plain nutritional value of the probiotic effect. Application of the live bacteria increased female and male pupal weight, various morphological traits of adult flies, including head width and thorax length, as well as survival (Yao et al. 2017). In the case of survival rate, autoclaved bacteria resulted in a greater increase compared to live bacteria (Yao et al. 2017).

Mramba and colleagues studied the effects of *E. sakazakii* on stable fly *Stomoxys calcitrans* development (Mramba et al. 2007). They observed that sterile media did not support any fly development and that the effect was reversed with the addition of *E. sakazakii*. However, an improved effect for fly development was mainly observed in cooperation with other microbial partners in non-sterilized media. The combination of non-sterile media and *E. sakazakii* resulted in a slight increase in the survival of larvae to the pupal stage, in pupal weight and adult emergence and also displayed increased duration of the larval and pupal stages compared to the non-sterile media without the inoculum with *E. sakazakii* (Mramba et al. 2007).

A similar probiotic effect was observed in transgenic diamondback moth larvae (Somerville et al. 2019). The larvae that were grown on an aseptic diet (sterile diet and addition of streptomycin) and were inoculated with streptomycin-resistant *E. cloacae* JJBC exhibited increased pupal weight and production of progeny compared to an aseptic diet without the inoculum (Somerville et al. 2019).

In *Apis mellifera jemenitica* Ruttner, however, the *E. kobei* strain that was examined for its probiotic effect did not manage to reduce mortality of bee larvae infected with *Paenibacillus* spores (Al-Ghamdi et al. 2018).

3.2. Enterobacter *and Protective Functions*

Insects live in close coalition with microorganisms, which immensely influence their ecology and evolution. Microorganisms, such as bacteria, archaea, fungi, protozoa, viruses, can be associated with their insect host permanently or transiently, and such

associations may be beneficial or harmful to the insects' fitness (Gurung et al. 2019). For instance, endosymbionts tend to be dependent on the hosts for obtaining nutrients, whereas they can provide fitness advantages in terms of nutritional components (see above), overcoming host defences, and protection from pathogens, parasites, or environmental stressors (Engel and Moran 2013; Mereghetti et al. 2017).

The insect microbiota are also able to provide protective functions to their hosts, thereby influencing the proliferation of important human or animal pathogens (Table 4), including *Plasmodium*, *Trypanosoma*, dengue, Zika, yellow fever or chikungunya viruses, inside the insect body with a variety of mechanisms, either indirectly, by causing innate responses of the insect immune system or directly, through competition for resources with the pathogen or the production of substances with anti-pathogenic effects (Dong et al. 2009; Moreira et al. 2009; Bian et al. 2010, 2013; Cirimotich et al. 2011b; Walker et al. 2011; Weiss and Aksoy 2011; van den Hurk et al. 2012; Zhang et al. 2013; Dennison et al. 2014; Frentiu et al. 2014; Aliota et al. 2016a, 2016b; Dutra et al. 2016; Tan et al. 2017; Kalappa et al. 2018).

Table 4. Enterobacter *species can provide protective functions to their hosts, aiding them to cope with abiotic and biotic stress*

Enterobacter Species	Strain Accession Number	Host	
Enterobacter sp.	JF690924	*Anopheles arabiensis* Patton	Mosquito
Enterobacter cloacae	Not available	*Anopheles stephensi* Liston	Mosquito
Enterobacter sp.	JQ680715	*Anopheles gambiae* Giles	Mosquito
Enterobacter cloacae, Enterobacter amnigenus	Not available	*Anopheles albimanus* Wiedemann	Mosquito
Enterobacter ludwigii	MF084966 MF084975	*Delia antiqua* (Meigen)	Onion fly
Enterobacter sp.	EU693561 EU693573	*Leptinotarsa decemlineata* Say	Colorado potato beetle
Enterobacter ludwigii, Enterobacter asburiae	KX398658 KX398648 KX398650 KX398654 KX398657	*Helicoverpa zea* (Boddie)	Corn earworm
Enterobacter agglomerans	Not available	*Rhagoletis pomonella* (Walsh)	Apple maggot fly
Enterobacter asburiae, *Enterobacter cloacae*, *Enterobacter* sp.	KT957438 KT957439 KT957440 KT957441 KT957442 KT957443	*Plutella xylostella* (Linnaeus)	Diamondback moth
Enterobacter sp.	KX117074	*Leptinotarsa juncta* Germar	False potato beetle
Enterobacter sp.	JX296530 KC977257	*Leptinotarsa decemlineata* Say	Colorado potato beetle
Enterobacter cloacae	KM878728 KM886372 KM878717	*Coptotermes formosanus* Shiraki	Formosan subterranean termite
Enterobacter cloacae	Not available	*Glyphodes duplicalis* Inoue et al.	Mulberry pyralid
Enterobacter amnigenus	Not available	*Anopheles dirus* Peyton & Harrison	Mosquito

These effects continuously draw attention as a means of controlling vector-based infectious diseases, like malaria or dengue virus, that are transmitted by *Anopheles* and *Aedes* mosquito vectors (Cirimotich et al. 2011a; Jayakrishnan et al. 2018; Saldaña et al. 2017).

3.2.1. Enterobacter *Anti-pathogenic Effects in Mosquitoes*

Among various bacterial strains that show anti-pathogenic effects, one of the best characterized is *Enterobacter* strain *Esp_Z*, which was isolated from the midgut of wild *Anopheles arabiensis* mosquitoes in Zambian populations. The *Enterobacter* strain inhibited *Plasmodium* development in the midgut by 98%, 99% and 99% before the ookinete, oocyst, and sporozoite stage respectively (Cirimotich et al. 2011c). This response proved to be independent of the mosquito immune system since anti-*Plasmodium* genes, that are usually triggered during *Plasmodium* infections, showed similar activity in mosquitoes infected with *Esp_Z* or with non-inhibitory bacteria.

Additionally, silencing of anti-*Plasmodium* genes with RNAi in mosquitoes infected with *Esp_Z* did not result in *Plasmodium* oocyst development (Cirimotich et al. 2011c). The response also proved to be independent of bacterial retention of mosquito factors that are essential for *Plasmodium* development like, xanthurenic acid, iron and substances involved in fatty acid metabolism. Moreover, the inhibition effect was dose-dependent, both *in vitro* and *in vivo* conditions, with 10^4 bacterial cells providing near-complete protection against parasite infection and coinciding with active bacteria replication (Cirimotich et al. 2011c).

In view of the above, the inhibition activity was therefore hypothesized to be the result of anti-pathogenic substances. Indeed, the *Enterobacter* strain was found to produce *Plasmodium*-killing (Luckhart et al. 1998; Kumar et al. 2003; Peterson et al. 2007; Molina-Cruz et al. 2008) reactive oxygen species (Cirimotich et al. 2011c). These molecules were not detected in non-inhibitory bacteria, and their effect was neutralized by antioxidants in *Esp_Z*-containing insects, such as vitamin C and glutathione (Cirimotich et al. 2011c).

On the other hand, Eappen et al. (2013) identified *Enterobacter cloacae* strains that inhibited *Plasmodium* development by activating a component of the immune system of *Anopheles stephensi* mosquitoes. More specifically, *E. cloacae* was able to induce the expression of a serine protease inhibitor (SRPN6) in the mosquito midgut. The effect was linked to bacteria that were acquired through feeding and were exposed to the luminal side of the midgut epithelium, whereas bacteria injected in the haemocoel and exposed to the basal surface of the midgut epithelium were incapable of SRPN6 induction (Eappen et al. 2013). Unlike strain *Esp_Z*, the *E. cloacae* strains did not interfere with *Plasmodium* ookinete formation, but with the differentiation of ookinetes into oocysts after they traversed the midgut epithelium. Silencing of the SRPN6 gene resulted in an increase in the number of *Plasmodium* oocysts and increased prevalence of infection, implying that additional immune components are likely to participate in the inhibition process by *E. cloacae* (Eappen et al. 2013).

In field-collected *Anopheles gambiae* mosquitoes from Cameroon, *Enterobacter* spp. bacteria isolated from their midgut reduced *Plasmodium falciparum* oocyst intensity and prevalence by 35% and 15%, respectively. However, the reduction in infection was lower when parasite exposure was higher (Tchioffo et al. 2013).

Enterobacter bacteria that were isolated from the midgut of field-collected *Anopheles albimanus* mosquitoes in Mexico suppressed *Plasmodium vivax* infection in the insectary and field-collected samples (Gonzalez-Ceron et al. 2003). After seven days of treatment, *E. cloacae* and *E. amnigenus* 2 reduced *Plasmodium* infection by 17 and 53 times compared to control mosquitoes, respectively. Additionally, *E. cloacae* infected mosquitoes showed 2.5 times lower *Plasmodium* oocyst density than the controls (Gonzalez-Ceron et al. 2003).

3.2.2. Enterobacter *Effects in Herbivorous Insects*

Two *Enterobacter ludwigii* strains (B424 and B539) that were part of the natural gut microbiota of the onion fly *Delia antiqua* collected from garlic fields in China, showed inhibitory effects against *Beauveria bassiana* (Balsamo), an entomopathogenic fungus that is used as a biocontrol agent in pest management applications (Valero-Jiménez et al. 2014; Zhou et al. 2019). The vital role of the bacterial symbionts of *D. antiqua* on the larvicidal potential of *B. bassiana*, was determined by first examining axenic (bacteria-free) and non-axenic larvae infected with the fungus (Zhou et al. 2019). In this case, treatment with the pathogen did not affect the survival of non-axenic larvae but indicated a significant reduction in the survival of axenic larvae. Furthermore, axenic larvae inoculated with microbiota were characterized by significantly higher survival rates than non-inoculated axenic larvae, when both were infected with the fungus (Zhou et al. 2019). Following the above tests, symbiotic bacteria were isolated from the surface and guts of the onion fly, and certain strains were tested for their anti-fungal effect. The *Enterobacter* strains strongly inhibited (ca. 70-99%) conidia germination and the hyphal growth of the entomopathogen fungus *B. bassiana*. Additionally, as expected from the previous tests, inoculation of axenic larvae with strain B424 significantly increased survival rate against the fungal infection (Zhou et al. 2019).

Similar observations were made with the Colorado potato beetle *Leptinotarsa decemlineata* fourth-instar larvae that were field-collected in Maryland and Virginia, USA (Blackburn et al. 2008). *Enterobacter* sp. strains 2B1C and 2B2D that were isolated from larval faecal fluid were found to inhibit two entomopathogens, *Photorhabdus temperata* Fischer-Le Saux et al. and the fungus *B. bassiana*. *P. temperata* is a bacterial symbiont of the entomopathogenic nematode *Heterorhabditis marelatus* Liu & Berry which can be used to suppress the Colorado potato beetle. However, in the presence of the *Enterobacter* strains the nematode fails to complete its reproductive cycle in the beetle, possibly due to the inhibition of *P. temperata* (Armer et al. 2004). The *in vitro* tests were based on comparing on PP3 agar plates (Bacto® Proteose Peptone) the growth of *P. temperata* and *B. bassiana* conidia in the presence of each *Enterobacter* strain. Both strains reduced the growth of *P. temperata* by approximately 33% while at the same time, strain 2B1C and 2B2D reduced fungal growth by almost 80% and 42.5% respectively (Blackburn et al. 2008).

Enterobacter strains have also been recognized as essential factors in shaping interactions between herbivorous insects and plants, by either activating or suppressing plant defences. In such a case of activation, laboratory-reared *Helicoverpa zea* larvae inoculated orally with an *Enterobacter ludwigii* strain were found to activate tomato plant *Solanum lycopersicum* defences (Wang et al. 2017). The strain was isolated from oral secretions, the regurgitant, of field-collected *H. zea* larvae in Rock Springs, Pennsylvania, USA. The *E. ludwigii* strain along with an *E. asburiae* strain were found to increase the activity of glucose oxidase (GOX), a molecular signal that induces plant defences in response to herbivory, in the labial glands of laboratory-reared larvae (Wang et al. 2017). Even though direct application of both strains on wounded tomato plants resulted in suppression of polyphenol oxidase (PPO) activity, a plant defence component which is regulated by jasmonic acid (JA), when tomato plants were damaged by *E. ludwigii*-inoculated larvae, they showed an increase in JA-regulated genes (proteinase inhibitor II (*Pin2*), cysteine proteinase inhibitor (*CysPI*) and polyphenol oxidase F (PPOF)), and a suppression in salicylic acid (SA) response (pathogenesis-related protein 1 (P4), *Pr1a* (P4)). Suppression of JA-mediated response proved to be dose-dependent, with high bacteria concentrations producing significant results when applied directly on the plant.

In the diamondback moth, two *Enterobacter* species, *E. asburiae* and *E. cloacae*, encoded all the genes related to the aerobic pathway for catechol degradation and could significantly degrade phenol *in vitro* (Xia et al. 2017). These compounds are important plant defence compounds of *Brassica* plants as they are toxic to insects (Xia et al. 2017).

In a contrary case of suppressing plant defences, in antibiotic-treated laboratory-reared false potato beetle (*Leptinotarsa juncta*) larvae, *Enterobacter* inoculation reduced JA-regulated gene expression (PPO) in tomato leaves (non-preferred host, *S. lycopersicum*), but did not have any effect on horsenettle (preferred host, *Solanum carolinense* Linnaeus) (Wang et al. 2016). Damage to leaves by untreated false potato beetle larvae or application of their oral secretions to wounded leaves resulted in reduced PPO activity in both plants compared to antibiotic-treated larvae (Wang et al. 2016). Additionally, direct application of *Enterobacter* on wounded leaves suppressed JA-regulated PPO and *CysPI* expression and increased SA-regulated *Pr1* expression in tomato, but not in horsenettle (Wang et al. 2016).

Similarly, Chung et al. (2013) observed that damage caused to tomato plants by antibiotic-treated Colorado potato beetle larvae increased JA-regulated gene expression (*CysPI* and *PPOF/B*) and a decrease in the SA defence response (*Pr1* (P4)) compared to untreated larvae. Additionally, lower *PPO* activity was observed when oral secretions of untreated larvae were applied on mechanically wounded plants. These observations suggested that bacteria in oral secretions of the Colorado potato beetle suppressed the JA-mediated defence mechanism. Indeed, an *Enterobacter* strain that was isolated from larval oral secretions suppressed JA-mediated antiherbivore defence response (PPO) in tomato plants when it was inoculated in antibiotic-treated Colorado potato beetle larvae (Chung et al. 2013).

4. BACTERIAL PARATRANSGENESIS WITH *ENTEROBACTER*

As *Enterobacter* strains form extremely stable relations with insects by constituting a prevalent portion of their gut microbiota, they can function as vectors for the introduction of functional genes that could be useful for multiple applications, including pest population control (Wilke and Marrelli 2015).

Such a system was examined in *Enterobacter cloacae* species isolated from the guts of Formosan subterranean termites in Hawaii (Husseneder and Grace 2005). The bacteria were transformed with a recombinant plasmid containing genes encoding ampicillin resistance and green fluorescent protein (GFP) and were fed to termite workers from three colonies (Husseneder and Grace 2005). The infection was established rapidly, with 90-100% of termite workers containing transformed bacteria 12 h after feeding, which persisted in the population for up to 11 weeks. Moreover, fluorescent microscopy revealed that the ingested bacteria expressed the GFP gene in the termite gut. Transformed bacteria were efficiently transferred through a colony, with infection rates reaching 80–100% after six days, even when the initial ratio between infected and uninfected termites was low. Recombinant bacteria were also transferred into the soil by infected termites but declined rapidly within four to five weeks (Husseneder and Grace 2005).

In another example, *Enterobacter cloacae* strain WBMH-3-CM^r was transformed with plasmids containing the ice nucleation gene *inaA* of *Erwinia ananas* IN10 and were ingested by larvae of mulberry pyralid *Glyphodes duplicalis* (Watanabe et al. 2000). Ice nucleation genes increase the supercooling points and reduce the tolerance of overwintering insects to cold, resulting in death by freezing. The supercooling points of mulberry pyralid larvae treated with the transgenic *E. cloacae* strain were higher than *E. ananas*-treated larvae, and their mortality rate reached 64.3% after 2 h exposure at -5°C and eventually increased to 95.2–100% after 6 and 18 h of exposure (Watanabe et al. 2000).

In *Anopheles dirus* larvae, *Enterobacter amnigenus* bacteria isolated from their gut tissue were transformed with the mosquito-larvicidal toxin of *Bacillus sphaericus* 2297 (Khampang et al. 2001). The fragment encoding the binary toxin was inserted into various plasmids, under different promoters in order to improve expression levels. *E. amnigenus* carrying a recombinant plasmid with the native *B. sphaericus* promoter exhibited the highest toxicity among the plasmids that were tested and proved to be 20 times more effective than *B. sphaericus* 2297 (Khampang et al. 2001).

5. INSECTICIDAL ACTIVITY OF *ENTEROBACTER*

Apart from their beneficial impact on host fitness as members of the insect gut microbiota, *Enterobacter* strains may also exhibit insecticidal activity even against their hosts, functioning as biocontrol agents in pest management applications (Table 5).

The shift in the behaviour of otherwise beneficial symbionts could be due to changes in the composition or location of the insect gut microbiota. Several examples of entomopathogenic *Enterobacter* strains have been documented. Among them, *E. cloacae* strain SL11 caused 30-73% mortality, depending on bacteria concentration, when fed to larvae of its host *Spodoptera litura* (Thakur et al. 2015). The strain also had a negative impact on essential fitness parameters of *S. litura* progeny, including development from larva to adult, growth rate, life span, morphology and reproduction. The pathogenic *E. cloacae* strain dominated the gut microbiota in infected insects, caused a reduction in the number of haemocytes and produced immune responses of phenoloxidase and lysozyme (Thakur et al. 2015).

Table 5. Enterobacter *species providing insecticidal activity*

Enterobacter Species	Strain Accession Number	Host		Function	Reference
Enterobacter cloacae	KP058541	*Spodoptera litura* (Fabricius)	Tobacco cutworm	Insecticidal effect	Thakur et al. 2015
Enterobacter hormaechei, Enterobacter sp.	GU906249 GU906251	*Rhynchites bacchus* (Linnaeus)	Leafroller weevil beetles	Insecticidal effect	Gokce et al. 2010
Enterobacter cloacae	Not available	*Bemisia argentifolii* Bellows & Perring	Silverleaf whitefly	Insecticidal effect	Davidson et al. 2000
Enterobacter cancerogenus	Not available	*Oberea linearis* (Linnaeus)	Hazelnut and walnut twig borer	Insecticidal effect	Bahar and Demirbağ 2007
Enterobacter sp.	KM979225	*Cimbex quadrimaculatus* (Müller)	Sawfly	Insecticidal effect	Cakici et al. 2015
Enterobacter sp.	JQ066777	*Spodoptera littoralis* (Boisduval)	Cotton leafworm	Insecticidal effect	Cakici et al. 2015
Enterobacter aerogenes	Not available	*Euproctis chrysorrhoea* (Linnaeus)	Brown-tail moth	Insecticidal effect	Yaman et al. 2000
Enterobacter aerogenes	AF306521	*Myrmeleon bore* (Tjeder)	Antlion	Insecticidal effect	Yoshida et al. 2001

Enterobacter strains with pathogenic effect against their host cause mortality in variable degree. In leafroller weevil beetles *Rhynchites bacchus* two *Enterobacter* strains (*E. hormaechei* Rb3 and *Enterobacter* sp. Rb5) caused 13% mortality (Gokce et al. 2010) in the silverleaf whitefly, *Bemisia argentifolii*, *E. cloacae* WFA73 produced 34% adult *B. argentifolii* mortality after 24 h and 75% after 48 h (Davidson et al. 2000). *E. cancerogenus* Ol11 caused 15% mortality in larvae of the beetle *Oberea linearis* ten days after treatment (Bahar and Demirbağ 2007).

The larvicidal effect of *Enterobacter* sp. CQ4 resulted in 58% mortality within ten days of third instar larvae of *Cimbex quadrimaculatus* (Çakici et al. 2015). In the cotton leafworm *Spodoptera littoralis*, *Enterobacter* sp. SL4 showed low larvicidal activity (17%) against third instar larvae, ten days after treatment (Çakici et al. 2014). Finally, an *Enterobacter aerogenes* isolate from the brown-tail moth *Euproctis chrysorrhoea* caused 45% mortality in third/fourth instar larvae (Yaman et al. 2000).

Yoshida et al. (2001) identified a mechanism of insecticidal activity based on a paralysing toxin that was produced by *Enterobacter aerogenes* bacteria contained in the saliva of *Myrmeleon bore* larvae. The 63 kDa protein assists larvae in capturing their prey and shows homology to the heat shock protein GroEL of *Escherichia coli*. The two proteins share similar sequences that contain only a few differences, in 11 residues and the carboxy terminus. A series of individual mutations revealed crucial residues for toxicity. Specific substitutions resulted in the loss of insecticidal activity of the *E. aerogenes* protein or the development of toxic effect by the otherwise harmless GroEL chaperone (Yoshida et al. 2001).

6. *ENTEROBACTER* GENOMICS

The number of available genome sequences from the *Enterobacter* taxon has not reached a threshold where comparative genomics can drive hypotheses and experiments. Recent progress in the genomics era resulted in one complete genome sequence of *Enterobacter cancerogenus* CR-Eb1 isolated from the larval gut of the greater wax moth *Galleria mellonella* (Chung et al. 2018). Also, there are several draft genome sequences of *Enterobacter* strains available from other insects (Table 3). In order to fully utilize the powerful analysis of comparative genomics and draft genomes from *Enterobacter* strains, more genomic and transcriptomic data are required from *Enterobacter* strains covering diverse functional roles. The availability of these genomes will be able to assist us in understanding their functional roles by defining their differences and similarities in gene content (Khamis et al. 2019).

The availability of *Enterobacter* sequenced bacterial genomes will also allow a more profound understanding of their organizational features that are related to fundamental cellular processes such as coordinated gene expression, chromosome replication and cell division. Genomic data will provide the means to characterize the fluidity of bacterial chromosomes, including genome rearrangements that imperil the selective features of chromosome order. Furthermore, a high-density microarray can be developed for the analysis of expression and genome content in a wide variety of *Enterobacter* strains, both sequenced and not sequenced.

Comparative genomics of more complete *Enterobacter* genomes will also allow studying the integration process from free-living to endosymbiont. Usually, symbiotic bacteria undergo drastic genetic, phenotypic, and biochemical changes, which can be detected by comparison with free-living relatives (Lo et al. 2016). Genome reduction, in some cases, is extremely strong, and it has generated the smallest bacterial genomes found to date; gene contents are so limited that their status as cellular entities is questionable (Latorre and Manzano-Marín 2017). It would be exciting to further improve our understanding of *Enterobacter* species' diversity in terms of their evolutionary history.

7. CONCLUSIONS

More than a hundred years of biological research has demonstrated the importance of microorganisms in the health and disease of higher organisms. Similarly, insects have symbiotic interactions that enable them to exploit unusually limited nutritional resources. In particular, recent findings suggest that symbiotic associations between insects and *Enterobacter* species may be beneficial to host fitness because of their various abilities to hydrolyse and ferment carbohydrates, catalyse nitrogen fixation, and produce vitamins and pheromones.

Also, the dominance of *Enterobacter* indicates an essential role in the protection of the insect host, or its nutritional resources, against parasitoids or predators, and also in terms of the interference in the transmission of malaria and other vector-borne diseases.

This review summarized our current knowledge of the relationship between *Enterobacter* and insect hosts. Characterization, exploitation and management of the insect-*Enterobacter* symbiotic associations can significantly contribute to and support integrated pest management applications for the control of agricultural pests and disease vectors.

To further decipher the *Enterobacter*-based symbiotic interactions we propose a systems biology approach in which, *in silico* predictions based on genomic analyses and phylogenetic information will be assessed by transcriptomic, proteomic, and metabolomic analyses. This information is considered essential for the success of downstream field applications.

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