GeneWatch UK further comment on Animal and Plant Health Inspection Service Docket No. APHIS–2014–0056: Environmental Assessment for the Field Release of Genetically Engineered Diamondback Moths

September 2014

GeneWatch UK is a not-for-profit organisation based in the United Kingdom. This is our second submission containing further comments on two aspects we did not cover in our first submission: (i) the issue of horizontal gene transfer (HGT) and its implications for antibiotic resistance; and (ii) the use of a multifunctional human viral domain from HSV1.

The risk of HGT provides an additional mechanism for the spread of antiobiotic resistance which should have been considered in the EA, alongside the risk of the selection of antiobiotic resistant gut bacteria in the GE diamondback moth (DBM) during mass breeding (considered in our first submission). Antiobiotic resistance in bacteria might have serious implications for human health, for example, consumption of the GE larvae by mice might risk the transfer of resistance to bacteria such as *Borrelia burgdorferi*, which causes Lyme disease. Doxycycline is the standard treatment for Lyme disease, so there is a risk that this could be rendered ineffective.

The implications of the use of HSV1 are unclear but raise some additional questions to be investigated.

Failure to correctly consider these potential risks are additional omissions from the environmental assessment (EA) which add further weight to our view that the EA is incomplete and that the application should be refused.

1. Horizontal gene transfer and implications for antibiotic resistance

1.1 Requirement to consider horizontal gene transfer

In our first submission we highlighted the EFSA Guidance which outlines the evidence that Oxitec would need to provide for its GE insects to be placed on the EU market (placing on the market means making available to third parties, whether in return for payment or free of charge).[1] This Guidance is relevant because it is the only guidance adopted worldwide for the risk assessment of GE insects and because Oxitec must supply an environmental risk assessment which meets EU standards prior to exporting GE diamondback moth eggs to the USA for open release (under Regulation 1946/2003/EC[2]). This notification must include a prior, existing environmental risk assessment which meets EU standards.

The EFSA Guidance includes Horizontal gene transfer (HGT) (Section 4.2.2), defined as "*any process in which an organism incorporates genetic material from another organism into its genome without being the offspring of that organism*". The Guidance states: "*The evaluation of the impact of HGT from GM insects includes analysis of the potential of exposure and transfer of recombinant DNA from GM insects and further dissemination to other organisms. Furthermore, if HGT can occur, the consequences of such transfer events for human and animal health and the environment must be evaluated*."

There is no specific guidance for the environmental assessment of GE insects in the USA. However, the importance of HGT is recognised in other international documents such as the proceedings of a joint IAEA/FAO technical meeting on GE insects held in 2002.[3] This notes that "*Concerns related to*

horizontal transmission and impact on biodiversity will be at the forefront of interactions between scientists, regulatory authorities and various interest groups".

1.2 Horizontal gene transfer and the piggyBac element

The gene transfer technology used by Oxitec relies on the *piggyBac* transposase. In relation to the *piggyBac* element, the IAEA/FAO proceedings state: "*The possibility remains for instability and horizontal transmission*" (page 10).

The USDA-APHIS-2008 report relied on in the EA cites concerns that HGT may pose a risk, particularly concerns raised by Handler (2004) that transgene movement into intermediary symbionts or infectious agents could occur during mass rearing, allowing rapid movement into other hosts after release. [4] The paper by Handler (2004) specifically cites concerns that the *piggyBac* transposon used by Oxitec to create its GE insects appears to have recently traversed insect orders by HGT and that this transposon has demonstrated the ability to transpose into an infectious baculovirus. USDA-APHIS-2008 dismisses these concerns on the basis that this has not been demonstrated outside the laboratory. However, this conclusion is out of date as Gilbert et al. (2014) has demonstrated[5]. These recent results provide strong support for the role of viruses as vectors of transposable element horizontal transfer between animals: the authors call for a systematic evaluation of the frequency and impact of virus-mediated horizontal transfer on the evolution of host genomes. In this paper, *PiggyBac* transposase was detected in insect viral genomes. Viruses appear to have the potential for greater rates of HGT than bacteria.[6] Other observations confirm the likelihood that inter-species movements of genes (and *piggyBac* in particular) can occur by viral vectors.[7,8]

The recent publication by Gilbert et al. (2014) demonstrates that, in the field, transposase can not only move from on insect genome to a baculovirus but also from the virus to another insect. This confirms Dr Handler's concerns, which were wrongly dismissed by USDA-APHIS-2008. The EA should have considered this new evidence and placed greater emphasis on the likelihood of HGT. We consider further below how HGT could pose risks to human health and the environment.

1.3 Oxitec's false claim that TTAV is negatively selected in bacteria

The company Oxitec considers HGT to micro-organisms, including the gut micro-biota of predators of the GE insects, on page 99 of the Oxitec report appended to the EA. However, this includes the false statement that: "*The inserted traits confer a strong negative selection, therefore it is unlikely that they would be capable of persisting in the gut flora of any predator or in the environment*". This statement is incorrect because it confuses the toxic phenotype conferred by overexpression of the tTAV in certain eukaryotes (mosquitoes, diamondback moth) and the primary drug-dependent transcriptional regulatory function of the TetR domain when it is expressed in bacteria.

Specifically, the tTAV allele inserted in the Diamondback Moth genome is composed of a N-terminal TetR domain; a functional bacterial transcriptional repressor fused to a VP16 trans-activating tegument protein derived from Human Herpes virus 1 (HSV1). In this configuration the TetR domain remains a functional bacterial tetracycline resistance determinant. In fact the TetR Gene is under strong positive selection in bacteria with a chromosomal copy of one of the Tet (A,B,C,D) efflux mechanisms.[9,10,11,12,13]

The killing mechanism due to overexpression of tTAV in some organisms is not fully understood and this adds additional uncertainties. [14]

1.4 Role of the tTAV allele as an antibiotic resistance marker gene (ARMG) for tetracycline and its derivatives

Since there are genuine concerns about horizontal gene transfer from the GE insects to micro-organisms and on to other hosts, it is important to be aware that antibiotic resistance could be transferred via this mechanism. This is a particular concern because tetracycline and its derivatives (oxytetracycline and doxycycline) are widely used in both medicine and agriculture.[15] Amongst many other uses, doxycycline is the standard treatment for Lyme disease (caused by infection with the bacteria *Borrelia burgdorferi*) which is spreading throughout New York State.[16] Lyme disease is transmitted to humans from a natural reservoir among rodents by ticks that feed on both sets of hosts.[17]

In the absence of the efflux system the TetR protein does not confer resistance to any antibiotics, but when the repressor gene is associated with the efflux mechanism in one loci they remain selected as a drug resistance cluster. [18] This occurs even in the absence of selective pressure through the use of the antibiotic tetracycline (concerns about the use of tetracycline in mass breeding were discussed in our earlier submission). Thus HGT may act as an additional mechanism for the spread of antibiotic resistance, with adverse implications for human health and the environment.

Lin et al. (2014) have recently demonstrated that the use of tetracycline during the breeding of Diamondback Moths selects for specific bacterial flora.[19] The risk of selecting for antibiotic resistant bacteria should therefore also have been considered in the EA.

For comparison, a scenario of transfer from tetracycline resistance determinants from GE crops to soil bacterial communities was studied by the FDA in 1996.[20] The FDA report argues that ARMGs for the antibiotics neomycin and kanamycin may be acceptable in GE plants because they are infrequently used antibiotics, neither is unique for any use, they are rarely are administered orally, and they are not used in agriculture or aquaculture to any great extent. Thus, the report argues, selective pressure would be minimal for development of resistant bacteria because the drugs are not used in humans or in animals to any great extent. The FDA report goes on to state: "*However, different circumstances may apply to other antibiotics. For example, with regard to the presence or absence of selective pressure, streptomycin and oxytetracycline may provide selective pressure in the environment because of their use as pesticides in agriculture*".

It is surprising that USDA-APHIS appears willing to allow the use of a tetracycline resistant marker gene in GE insects, when this clearly contradicts the industry guidance for GE plants issued by the FDA. This issue has not been dealt with correctly in the EA and should have led to refusal of the application.

1.5 Summary of concerns about Horizontal gene transfer (HGT) and implications for antibiotic resistance

In summary:

- The EA has failed to take account of recent evidence that highlights the potential for horizontal transfer of the genetic construct from the GE insects to viruses and other organisms;
- The EA has wrongly relied on a false statement from Oxitec that claims that any transferred genetic trait would be under negative selection: in reality positive selection of the antibiotic resistant trait can be expected in bacteria;

- The EA has failed to take account of FDA Guidance on the use of antibiotic resistant marker genes that warns against the use of markers resistant to antibiotics widely used in agriculture, such as tetracycline and its derivatives.

These important failings add weight to the arguments in our previous submission that the application should be refused.

2. The use of a multifunctional human viral domain from HSV1

It is important to consider tTAV as a chimeric protein that results from the fusion of a bacterial resistant determinant with Herpes virus derived tegument protein C-terminal domain. The human herpes virus 1 VP16 tegument protein nomenclature is synonymous with; UL48, alpha TIF, ICP25, Vmw65. The BLAST-P protein sequence homology analysis shows 100 % identity between the tTaV and HSV1 VP16 (residues 363 to 490). The VP16 C–terminal acidic region of viral tegument protein confers to the TetR-VP16 fusion (tTA or tTaV) the transcription activator capacity in eukaryotes. The DNA sequence encoding the gene is an exact match to the human viral gene.

The implications of the use of HSV1 are unclear but raise some additional questions to be investigated.

As result of selective pressure, VP16 is a complex multifunctional protein which is also a key tegument structural component of HSV1 virus. The C-terminal transactivation domain of VP16 present on tTAV is also a VP22 interacting domain. HSV1 tegument assembly involves the interaction of the C-terminal domain of VP16 with VP22 and the VP22/VP16 protein interacting residues are well conserved among many herpes viruses.[21,22,23,24,25,26,27] This suggests that VP16 can interact with a large family of VP22 and we can conclude that the viral domain present in the tTAV allele of the GE Diamondback Moth (or any other tTaV transgenic organism) conserves its capacity to interact with major viral tegument VP22 protein originating from wide variety of herpes viruses. The VP16 parental organism herpes virus is a pathogen that infects 30 to 40 % of human population. The domain used in the tTAV retains not only the regulatory function but moreover the minimal structural element to recruit herpes virus tegument protein VP22.

It is clear that the DBM will not be infected by HSV1 but if VP16 moves to any species that can host herpes, VP16 would interact with its VP22 homologues.

This gives rise to two potential risks:

(i) ***Can the tTAV allele be transduced to another host?*** This might occur if the tTaV allele is transferred from insects to vertebrates by HGT. The tTaV (TetR-VP16) protein present during herpes virus cycle (infection and assembly) will be recruited by VP22 protein and could be packaged as part of the tegument. The assembled herpes virus could potentially transduce the tTAV protein to the new host.

(ii) ***What is the likelihood and hazard of trans-complementation between the Vp16 domain and the herpes virus?*** Trans-complementation is a process in which a viral protein, often expressed from an integrated transgene, supports or enhances infection by an invading virus. Concerns about trans-complementation have previously been raised in the context of GE plants.[28,29] Questions remains about the risk of trans-complementation when a gene or part of viral gene derived from human pathogens is used in GE insects.

In summary, USDA-APHIS has not considered any potential risks associated with the use of a multifunctional human viral domain from HSV1 in the genetic construct. These issues should have

been included in the EA. In particular, regulators must actively consider and determine the question of whether the active domain of a human viral chimeric gene should be released in the environment as part of genetically engineered insect, and this issue should have been part of the public consultation.

3. Conclusion

The EA has failed to correctly consider the risks associated with:

i) the issue of horizontal gene transfer (HGT) and its implications for antibiotic resistance and;

ii) the use of a multifunctional human viral domain from HSV1.

These are additional omissions from the environmental assessment (EA) which add further weight to the view expressed in our first consultation response that the application should be refused.

For further information contact:
Dr Helen Wallace
Director
GeneWatch UK
60 Lightwood Rd
Buxton
SK17 7BB
UK
Email: helen.wallace@genewatch.org
Tel: +44-(0)1298-24300
Website: www.genewatch.org

References

[1] EFSA (2013) Guidance on the environmental risk assessment of genetically modified animals. EFSA Journal 2013;11(5):3200 [190 pp.]. http://www.efsa.europa.eu/en/efsajournal/pub/3200.htm

[2] REGULATION (EC) No 1946/2003 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 15 July 2003 on transboundary movements of genetically modified organisms. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2003:287:0001:0010:EN:PDF

[3] Status and risk assessment of the use of transgenic arthropods in plant protection Proceedings of a technical meeting organized by the Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture and the Secretariat of the International Plant Protection Convention, held in Rome, 8 to 12 April 2002. IAEA-TECDOC-1483. March 2006. http://www-pub.iaea.org/mtcd/publications/pdf/te_1483_web.pdf

[4] Handler AM (2004) Understanding and improving transgene stability and expression in insects for SIT and conditional lethal release programs. *Insect Biochemistry and Molecular Biology*, **34**(2), 121–130.

[5] Gilbert C, Chateigner A, Ernenwein L, Barbe V, Bézier A, Herniou EA, Cordaux R (2014) Population genomics supports baculoviruses as vectors of horizontal transfer of insect transposons. *Nature Communications*, **5**: 3348. doi:10.1038/ncomms4348

[6] Keese P (2008). Risks from GMOs due to horizontal gene transfer. *Environmental Biosafety Research*, **7**(3), 123–149.

[7] Thézé J, Cabodevilla O, Palma L, Williams T, Caballero P, Herniou E (2014) Genomic diversity in European *Spodoptera exigua* multiple nucleopolyhedrovirus isolates. *Journal of General Virology*, **95**(10), 2297-2309.

[8] Jehle JA, Nickel A, Vlak JM, Backhaus H (1998) Horizontal escape of the novel Tc1-like lepidopteran transposon TCp3.2 into *Cydia pomonella* granulovirus. *Journal of Molecular Evolution*, **46**(2), 215–224.

[9] Hillen W, Berens C (1994). Mechanisms underlying expression of Tn10 encoded tetracycline resistance. *Annual Review of Microbiology*, **48**, 345–369.

[10] Glenn LM, Lindsey RL, Folster JP, Pecic G, Boerlin P, Gilmour MW *et al.* (2013) Antimicrobial Resistance Genes in Multidrug-Resistant Salmonella enterica Isolated from Animals, Retail Meats, and Humans in the United States and Canada. *Microbial Drug Resistance*, **19**(3), 175–184.

[11] Berens C, Hillen W (2003) Gene regulation by tetracyclines. Constraints of resistance regulation in bacteria shape TetR for application in eukaryotes. *European Journal of Biochemistry / FEBS*, **270**(15), 3109–3121.
[12] Eckert B, Beck C F (1989) Overproduction of transposon Tn10-encoded tetracycline resistance protein results in cell death and loss of membrane potential. *Journal of Bacteriology*, **171**(6), 3557–3559.
[13] Nguyen TN, Phan QG, Duong LP, Bertrand KP, Lenski RE (1989). Effects of carriage and expression of the Tn10 tetracycline-resistance operon on the fitness of *Escherichia coli* K12. *Molecular Biology and Evolution*, **6**(3), 213–225.
[14] Schetelig MF, Handler AM (2012). Strategy for enhanced transgenic strain development for embryonic conditional lethality in *Anastrepha suspensa*. *Proceedings of the National Academy of Sciences of the United States of America*, **109**(24), 9348–9353.
[15] Auerbach EA, Seyfried EE, McMahon KD (2006) Tetracycline resistance genes in activated sludge wastewater treatment plants. Water Environment Foundation. http://www.environmental-expert.com/Files%5C5306%5Carticles%5C8866%5C117.pdf
[16] https://www.health.ny.gov/diseases/communicable/lyme/
[17] Tilly K, Rosa PA, Stewart PE (2008). Biology of Infection with Borrelia burgdorferi. *Infectious Disease Clinics of North America*, **22**(2), 217–234.
[18] Berg CM, Liu L, Wang B, Wang MD (1988). Rapid identification of bacterial genes that are lethal when cloned on multicopy plasmids. *Journal of Bacteriology*, **170**(1), 468–470.
[19] Lin X-L, Kang Z-W, Pan Q-J, & Liu T-X (2014) Evaluation of five antibiotics on larval gut bacterial diversity of Plutella xylostella (Lepidoptera: Plutellidae). *Insect Science*. doi:10.1111/1744-7917.12168
[20] FDA (1998) Use of Antibiotic Resistance Marker Genes in Transgenic Plants. Guidance for Industry Use of Antibiotic Resistance Marker Genes in Transgenic Plants Draft Guidance. September 4, 1998. http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Biotechnology/ucm096135.htm
[21] Wysocka J, Herr W (2003) The herpes simplex virus VP16-induced complex: the makings of a regulatory switch. *Trends in Biochemical Sciences*, **28**(6), 294–304.
[22] Xing J, Ni L, Wang S, Wang K, Lin R, Zheng C (2013) Herpes Simplex Virus 1-Encoded Tegument Protein VP16 Abrogates the Production of Beta Interferon (IFN) by Inhibiting NF-κB Activation and Blocking IFN Regulatory Factor 3 To Recruit Its Coactivator CBP. *Journal of Virology*, **87**(17), 9788–9801.
[23] Elliott G, Mouzakitis G, O'Hare P (1995) VP16 interacts via its activation domain with VP22, a tegument protein of herpes simplex virus, and is relocated to a novel macromolecular assembly in coexpressing cells. *Journal of Virology*, **69**(12), 7932–7941.
[24] Hafezi W, Bernard E, Cook R, Elliott G (2005) Herpes Simplex Virus Tegument Protein VP22 Contains an Internal VP16 Interaction Domain and a C-Terminal Domain That Are Both Required for VP22 Assembly into the Virus Particle. *Journal of Virology*, **79**(20), 13082–13093.
[25] O'Regan KJ, Murphy MA, Bucks MA, Wills JW, Courtney RJ (2007) Incorporation of the herpes simplex virus type 1 tegument protein VP22 into the virus particle is independent of interaction with VP16. *Virology*, **369**(2), 263–280.
[26] Blondeau C, Marc D, Courvoisier K, Vautherot J-F, Denesvre C (2008) Functional Homologies between Avian and Human Alphaherpesvirus VP22 Proteins in Cell-to-Cell Spreading as Revealed by a New cis-Complementation Assay. *Journal of Virology*, **82**(18), 9278–9282.
[27] Dorange F, El Mehdaoui S, Pichon C, Coursaget P, Vautherot JF (2000) Marek's disease virus (MDV) homologues of herpes simplex virus type 1 UL49 (VP22) and UL48 (VP16) genes: high-level expression and characterization of MDV-1 VP22 and VP16. *The Journal of General Virology*, **81**(9), 2219–2230.
[28] Podevin N, du Jardin P (2012) Possible consequences of the overlap between the CaMV 35S promoter regions in plant transformation vectors used and the viral gene VI in transgenic plants. *GM Crops & Food*, **3**(4), 296–300.
[29] Latham JR, Wilson AK (2008) Transcomplementation and synergism in plants: implications for viral transgenes? *Molecular Plant Pathology*, **9**(1), 85–103.