Report on the outcomes of a Virtual Mobility[[1]](#footnote-1)

Action number: CA18221

Grantee name: Frances Orton

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| **Virtual Mobility Details**  Title: Extrapolate long-term, breeding output effects from developmental testing in amphibians  Start and end date: 03/01/2024 to 14/01/2024 |
| **Description of the work carried out during the VM**  Description of the virtual collaboration and activities carried out during the VM, with focus on the work carried out by the grantee. Any deviations from the initial working plan shall also be described in this section.  *(max. 500 words)*   * Review of current amphibian OECD test guideline’s and extended AMA protocols (Ortego et al. 2021). * Review of available literature of relevance to the aims of this VM. * Provide recommendations for updating the test guidelines and methods to assess health of wild amphibians. * Deliver the report (see below). |
| **Description of the VM main achievements and planned follow-up activities**  Description and assessment of whether the VM achieved its planned goals and expected outcomes, including specific contribution to Action objective and deliverables, or publications resulting from the VM. Agreed plans for future follow-up collaborations shall also be described in this section. |
| *(max. 500 words)*  **ACTION SCIENTIFIC OBJECTIVES**   1. To identify an improved testing framework and endpoints for pesticide ERA for amphibians indicating adversity. 2. To propose innovative strategies for implementing ERA schemes for amphibians based on increasing representativeness and ecological relevance of the assessment while minimising animal use. 3. To design an ERA scheme, targeting a pesticide usage safe for amphibians, that can be further implemented by EU and national authorities across Europe and serve as a reference to authorities responsible for ERA around the globe. 4. To design a protocol for post-registration monitoring of pesticide risks to amphibians.   **REPORT**  The World Health Organisation (WHO) definition for adversity (WHO/IPCS, 2009) is:  ‘A change in the morphology, physiology, growth, development, reproduction or life span of an organism, system or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress or an increase in susceptibility to other influences’.  Adversity is assumed in the absence of appropriate scientific data demonstrating non-relevance of alterations in any endpoints pertaining to growth, development or reproduction. This is because effects on growth, development and/or reproduction are generally regarded as relevant for the maintenance of wild populations and thereby are assume to translate into effects at the population level (Kortenkamp et al., 2011). Behavioural changes and ‘impaired ability to cope with additional stress’ are factors implicitly covered by the WHO definition of adversity, since they would affect the reproductive performance and development – but these are generally poorly defined.  For anurans amphibians, the timing of larval development into the adult form and the size at metamorphosis completion are delicately balanced phenomena. There is an impetus to complete metamorphosis rapidly due to the high predation risk within the aquatic environment, but as metamorphosis is an energetically expensive life event, developmental rates are balanced against energetic availability. Ultimately, optimal aquatic conditions result in large metamorphs that complete metamorphosis rapidly and a positive correlation between body mass and developmental stage are expected in unimpacted/healthy populations (Rose, 2005). Although it has been demonstrated that data derived from fish toxicity tests are generally protective for larval anuran amphibians (Glaberman et al., 2019), as fish do not undergo metamorphosis, it is unknown whether these data are indeed protective for natural amphibian populations. Indeed, one recently published study reporting the effects of polluted water exposure *ex situ* on natterjack toad (*Epidalea calamita*) growth and development, reported no effects on body mass or snout-vent length of the sub-sampled tadpoles, however, metamorphs deriving from larvae reared in water collected from polluted sites were under-sized (Macleod et al., 2024).  It is well known that size at metamorphosis is an important facet determining survival of juveniles to sexual maturity; thereby with potential to impact population stability and several older studies demonstrated this (Berven, 1990; Rose, 2005; Shine, 1979). More recently, it was reported that the sub-optimal water conditions for larvae reared in water from polluted test sites may have contributed to inadequate fat reserves needed for undergoing metamorphosis, resulting in under-sized metamorphs (Crespi and Warne, 2013). In addition to survival and metamorph size, indirect effects have also been reported/ For example, size at metamorphosis of laboratory reared cane toads (*Rhinella marinus*) determined prey acquisition success (Cabrera-Guzmán et al., 2013). In laboratory reared *Rana latastei*, smaller size at metamorphosis resulted in shorter legs and poorer jumping performance, with potential impacts for predator avoidance (Francesco Ficetola and De Bernardi, 2006). Further, in wild red-spotted Argentina frogs (*Argenteohyla siemersi pederseni*), size at metamorphosis determined age at sexual maturity (Cajade et al., 2013).  In addition to size at metamorphic completion, changes in the timing of metamorphosis are considered population relevant effects, as alterations to this endpoint clearly demonstrate effects on development. However, it should be borne in mind that the degree of delay or acceleration in the development that can be considered adverse at population level is uncertain (Marty et al., 2017). As thyroid histopathology often represents compensation to lowered thyroid hormone levels, effects on this endpoint alone should not be considered as ‘adverse’ (Marty et al., 2017). Behavioural responses are also known to be sensitive to pollutants (Sievers et al., 2019), however, the potential of these endpoints to inform on adversity is poorly understood.  With regards to the amphibian OECD test guidelines, using the Amphibian Metamorphosis Assay (AMA), it is possible to assess the rate of metamorphosis and a delay in this endpoint could conceivably be deemed an ‘adverse’ effect, as in the wild, this may result in a reduced chance of survival. The better-established endpoints for indicating adversity are time to metamorphosis completion and size at metamorphosis, neither of which can be assessed with the AMA. These endpoints are both assessed in the LAGDA (Larval Amphibian Growth and Development Assay: both at larval stage NF 62 and at completion of metamorphosis, NF stage 66) and therefore the LAGDA provides evidence of adverse effect in this regard, whereas the AMA does not.  A new test guideline has recently been proposed by Ortego *et al.* (2021) as an alternative to the AMA – termed the “Extended Amphibian Metamorphosis Assay (E-AMA)”. One difference between the AMA and the E-AMA is fixed-stage *versus* fixed-time. The rationale for this is that inter-individual variation in timing of metamorphosis will naturally occur in a population to reduce predation pressure – therefore a fixed-time design increases variability within controls and exposed organisms due to the necessity to stage-match sampled animals. Ultimately, the high variability observed using a fixed-time design results in a larger *n* being required. In particular, chemicals with thyroid-disrupting mode of action cause dysregulation in metamorphic development, resulting in a low concordance between control *versus* treated organisms and a low *n* that can be compared.  The authors also propose that the E-AMA could be used instead of the LAGDA, for assessing adversity. However, additional endpoints assessed in the LAGDA include time to metamorphosis, size at metamorphic completion, detoxification and reproductive endpoints (Table 1). The E-AMA test, in contrast to the AMA, does include time to reach NF stage 62 and the body mass achieved by the larvae at this stage. However, as the test is terminated at this point the degree to which delay at NF stage 62 can predict delay to completion of metamorphosis (i.e. NF stage 66) is unknown, as is the degree to which body mass or snout-vent length measured at NF stage 62 can predict effects on these endpoints at completion of metamorphosis (NF stage 66). Therefore, the utility of measurements taken at NF stage 62 to indicate adversity require further investigation. These data could be obtained by extending the E-AMA to NF stage 66 during the ring test phase. This would allow an assessment of the utility of data collected at stage 62 and whether these are indeed indicative of those collected at stage 66. If these measurements are shown *not to be* indicative of adverse effects – defined as size at metamorphosis completion and time to metamorphosis – then this would provide a rationale for extending the E-AMA to include NF stage 66. Further, the inclusion of behavioural endpoints – which can be more directly linked to adversity – such as altered swimming/feeding rates, should be considered.  Behavioural changes or impaired ability to cope with additional stressors which have the potential to impact the population stability of non-target organisms are included within the definition of adversity. For amphibian test guidelines, endpoints such as the presence of tadpole abnormalities, or other effects that have the potential to impact on chances of survival, such as swimming behaviour, are not well defined. Indeed, in both the AMA and the LAGDA it is stated that: “Cases of abnormal behaviour and grossly visible malformations and lesions should be recorded”; with some examples given for those that can indicate overt toxicity provided; but without standard criteria etc. Although using behavioural endpoints presents challenges, for example, ensuring appropriate training of technicians, standardisation of recording as well as establishing the behavioural changes which can be best linked to adverse population relevant effects; there is evidence that pollutants impact behaviour during the larval phase, particularly swimming activity (for review see: Sievers et al., 2019). These additional effects could be incorporated within existing test guidelines – importantly, this can be done without increasing *n*.    Table 1. Primary (●) and optional (○) endpoints and timing of measurements in the amphibian metamorphosis assay (AMA) and the larval amphibian growth and development assay (LAGDA) according to OECD TG 231 (AMA) and 241 (LAGDA). Adapted from Ortego et al. 2021.    a Non-specific for thyroid.  b Non-specific for thyroid unless accompanied by significant thyroid histopathology  \* Phenotypic sex only (based on gonad morphology).  \*\* Overt and significant changes in apical endpoints indicating developmental acceleration or asynchrony may preclude the necessity to perform histopathological analysis of the thyroid glands. However, thyroid histology may be required by some regulatory authorities regardless of the apical responses.  ***CONCLUSIONS AND RECOMMENDATIONS***  Body mass at completion of metamorphosis is currently the endpoint with the most evidence for being applicable to indicate adversity, however, as the LAGDA is performed very rarely, these data are absent for nearly all registered substances. Altered timing of metamorphosis, too, has the potential to induce population-level effects, however, there are a wide range of possible outcomes and these are poorly understood. For example, accelerated metamorphosis can result in lower weight and size, which reduces individual fitness and could affect later survival under natural conditions. Conversely, delay or failure of metamorphosis, might cause an increase or complete mortality of a tadpole population in the environment due to an inability to respond to a change in pond status (drying up, freezing, etc.). It has been proposed that population models should be used to determine the percentage effect observed in a laboratory toxicity test that is needed to exceed a threshold percentage that translates into a reduction in population recruitment, size, or stability across a representative “worst-case” landscape (Crane et al., 2019). Development of population modelling may be a practical approach to making judgments of likely population effects in the absence of definitive data. For this, a much greater understanding of fundamental biology of a wide range of species is required. In particular, comparative studies of *X. laevis* to species representing a range of amphibian families would be beneficial.  With regards to reproductive toxicity, there are very few data as the LAGDA is the only test to address this and it is rarely performed. Furthermore, very few lifecycle studies in amphibians have been conducted (but see: Gyllenhammar et al., 2009; Karlsson et al., 2021; Kvarnryd et al., 2011; Orton et al., 2018; Pettersson et al., 2006; Porter et al., 2011) so little is known regarding potential long-term consequences of chemicals in amphibians. Within the LAGDA, early-life exposure effects on adult reproduction are not assessed and therefore this is a major gap in risk assessment for amphibians; with the relative sensitivity of amphibians compared to other wildlife (e.g. fish) unknown. To this end, it has been suggested that the model species used for OECD testing should be *Silurana tropicalis* instead of *X. laevis*, as *S. tropicalis* develops more rapidly, reaching reproductive maturity earlier (Mitsui et al., 2006). Limited evidence also suggests that this species displays similar sensitivity to thyroid disruptors (Mitsui et al., 2006). Overall, the OECD amphibian test guidelines are not currently well designed to assess adverse effects of chemicals for amphibian individuals or their populations.  ***Recommendations***   * Body mass at metamorphic completion and time to metamorphosis should be tested against measurements taken at NF stage 62 to investigate concordance, preferably during the ring-test design stage for the E-AMA with adjustments to the AMA/E-AMA made as needed. * Optimisation of the measurement of behavioural endpoints, such as altered swimming and/or feeding rates, should be undertaken for possible inclusion within test guidelines, depending on the relative sensitivity of these endpoints compared to morphological endpoints. * Reproductive toxicity should be assessed in amphibians using *S. tropicalis* for a few selected compounds, to compare these data with fish life-cycle data, to gain understanding of the degree to which fish derived data are protective for amphibians. Further, endpoints recorded during the larval stages, such as gonadal histopathology or gonado-somatic-index should be assessed for their accuracy/applicability as predictors of the observed reproductive toxicity endpoints (such as fertility, offspring fitness) measured in sexually mature adults. * Evidence/endpoints optimised for chemicals testing could potentially be utilised to assess the health of wild amphibian populations. |

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1. This report is submitted by the grantee to the Action MC for approval and for claiming payment of the awarded grant. The Grant Awarding Coordinator coordinates the evaluation of this report on behalf of the Action MC and instructs the GH for payment of the Grant. [↑](#footnote-ref-1)