CA16224 - European Raptor Biomonitoring Facility
Working Group 1 & 2 Workshop
Madrid, Spain, 24-26 April 2019

Risk assessment of Anticoagulant Rodenticides in European Raptors

WORKSHOP REPORT

Organising Committee
Antonio J. García-Fernández, Lead WG2
Richard Shore, Lead WG1
Philippe Berny, WG2
Rafael Mateo, WG2

Local Organisers
Rafael Mateo. IREC-CSIC-UCLM
National Museum of Natural History in Madrid, Spain.

E-mail: Rafael.Mateo@uclm.es

INTRODUCTION AND OBJECTIVES

This meeting was convened by WGs1&2 at the National Museum of Natural History, Madrid, Spain.

In the Memorandum of Understanding of ERBFacility, and concretely among the aspects related to the "Analysis Arena" (WG1&2), it appears as a key element the necessity of a networking and coordination among ecotoxicologists and analytical laboratories, decision-makers and regulatory agencies. Indeed, this objective will be achieved piloting joint assessment and reporting between collaborating labs to deliver proof of concept.

At the end of the second period and during all the 3rd period is expected that WG1&2 will work to build a network of collaborating laboratories capable of delivering pan-European surveillance and monitoring key priority pollutants under Biocides directives like anticoagulant rodenticides (ARs). For these specific tasks (Tasks 1.3, 2.3), WG1&2 proposed establishing activities regarding quality control and potential for sample exchange between laboratories and collections.

This workshop aims to address several issues related to risk assessment of exposure to anticoagulant rodenticides in European raptors, including: usefulness of certain raptor species to monitor rodenticides based on land use and human activities; secondary exposure in predators; potential sublethal effects due to chronic exposures; review of analytical methods among labs; usefulness of exposure and effect biomarkers.

The issues cited above have been organized in four specific topics, which will be discussed during the following four respective sessions: 1) Anticoagulant Rodenticides and their applicability to the Proof of Concept designed in the Stirling meeting; 2) Interlaboratory comparison with special attention to the quality control procedures to guarantee reliable results; 3) Forensic tools to facilitate diagnosis of anticoagulant rodenticide secondary poisonings, and 4) Propose aims, requirements, and expected results regarding to a short term scientific mission about anticoagulant rodenticides.

WORKSHOP 1st SESSION: INTRODUCTORY PRESENTATION AND PLAN FOR THE WORKSHOP
(Richard Shore, Antonio García-Fernández, Rafael Mateo)

During the first session, local organiser Rafael Mateo welcomed the assistants and explained logistical questions.

Richard Shore and Antonio Juan García-Fernández (Lead WG1) introduced the ERBFacility COST Action, especially for the new participants, giving an overview of the project, to remind the objectives, the role and work of Working Group 1 & 2 and explained the scope and objectives of the workshop itself.

WORKSHOP 2nd SESSION: RODENTICIDES FOR PROOF OF CONCEPT (Rafael Mateo)

This session was devoted to discuss about the selection of compounds for the development of a proof of concept for the assessment of anticoagulant rodenticides exposure in birds of prey from Europe.

To achieve the purpose of the selection of compounds, first of all, Prof. Richard Shore explained the main outputs of the WG4 Meeting hold in April in Stirling. Afterwards, Prof. Phillipe Berny focused on the description of the main rodenticides used in Europe (including non anticoagulant) and the measures that can be hold for risk mitigation.

After the coffee break, the criteria for the selection of compounds were briefly discussed. The criteria chosen were *Regulatory*, *Analytical*, *Commercial*, *Scientific*, *Toxicity* and *Epidemiologic*. Then, small groups were made to discuss which advantages and disadvantages presented the anticoagulant rodenticides in terms of these criteria. Based on this, the existing compounds would be selected or dismissed for the proof of concept. The detailed information is provided in document 1. To sum up, those compounds not currently registered in Europe (i.e. warfarin, coumatetralyl…) were ruled out at first step and not discussed for the rest of criteria. In regards to the commercial criteria, the approximate number of products registered for each compound was indicated, as this is very probably linked to the frequency of detection and degree of exposure in the animals studied, although the degree of persistence may also affect. Those compounds considered highly toxic (i.e. bromadiolone, brodifacoum…). Regarding the analytical point of view, most compounds can be analysed although the methods are not standardised yet.

Based on the criteria discussed by all the participants, seven compounds were selected for the "Proof of concept": coumatetralyl, bromadiolone, brodifacoum, difenacoum, difethialone, flocoumafen and chlorophacinone.

WORKSHOP 3rd SESSION: INTERLABORATORY COMPARISON - QC: (Pilar Gómez, Antonio, Rafa, Richard)

During the first part of 3rd session, all the participants contributed to make a list of laboratories that could be involved in the proof of concept, as they are known to be able to analyse the anticoagulant rodenticides compounds selected during the 2nd session. As some information about the potential laboratories was missing, this list could not be finished during the workshop. Hence, it was decided that the list would be sent by Pilar Gómez-Ramírez to circulate among the participants of the workshop to be fulfilled within 15 -20 days.

For the second part of 3rd session, the participants of the workshop were split in three working groups led by Richard Shore, Phillipe Berny and Pilar Gómez-Ramírez, so that each group would discuss about certain aspects to be considered for the proof of concept of anticoagulant rodenticides. After the discussion in groups, all the considerations were explained by each lead and discussed with all the participants of the workshop. The aspects considered are explained in detail below:

Quality control criteria:

For a suitable interlaboratory comparison, certain quality control (QC) criteria should be fulfilled by participant laboratories. Thus, and those QC criteria were discussed (led by Pilar Gómez) so that the laboratories that may participate in the comparison should meet them (see document). Most of

these QC criteria can be based on some reference documents such as the guide SANCO, EU Directives or certain forensic documents:

- Method validation parameters: Minimum values of recoveries, reproducibility and repeatability should be set.
- Certain limits of detection and quantification may also be set, and the methods and criteria used to calculate them will be considered.
- Data about the uncertainties and specificity of the method are necessary.
- Other aspects such as the confirmation criteria, the need to check for cross-contamination and how to avoid it during processing of samples (specially in during postmortem manipulation) and the stability of standards were also pointed out.
- Finally, it was also considered necessary to have reference material, as this is not available by now, and it was discussed about the need to use internal standards.

Comparability of analytical methods

Another point of discussion in working groups was about the comparability of the existing analytical methods to be used in the proof of concept and how to interpret the results already obtained by different methods (led by Richard Shore). This is a relevant point as the different results that will be obtained by each participant laboratory should be comparable, which implies certain harmonization and detailed information about the methodologies used. In this sense, the participants indicated the following issues to consider:

- Information about the animals under study and sampling methods:
    - Cause of death and other necropsy findings
    - Information on age class
    - Body condition of animal may be a variable to study as it may be either an effect of anticoagulant exposure or it may influence the liver concentrations
    - Information about the number of samples and number of individuals included in the studies should be indicated.
    - It should be specified if the concentrations reported refer to individuals or are averaged for categories such as age, species, etc.
    - Issues of using single data points per square or multiple data points and expressing the uncertainty, or use all the data and run MC simulations to give values per square
    - Continuity of sample collection is also important to understand for people providing data. There is an interest to know if similar data may be provided by them in the future
- Information about sample processing:
    - To know if the samples may be dehydrated and if that was accounted for.
    - To know if the whole liver was homogenised or only a part taken. This may be relevant as it is unknown if there is significant variation within the liver in residue concentration (variation between lobes for example
- Information about the analytical methodology:
    - The units used to report rodenticide concentrations should be homogenized to be able to compare them among different studies
    - Regarding the limits of detection and quantification, it was suggested that a common level may be set. In addition, there should be information about the methods used to set these limits and if the limits refer to instrumental or sample analyses.
    - A common point of discussion regarding analytical methods refers to the values assigned to non-detected compounds in the samples analysed.

- Methodology for extraction and clean-up of biological samples should be described in detail, including the reagents used.
- To indicate if blanks are being used and if data are being corrected for blanks.
- It is important to indicate the % of recoveries obtained for each compound, how they are calculated (using spiked blanks, internal standard or RI) and if results are being corrected for recoveries.

Interlaboratory comparison

Another group, led by Phillipe Berny, focused on the requirements to do an interlaboratory comparison among selected laboratories.

- Preparation of samples: Since there is no reference material for analysis of anticoagulant rodenticides, a big pool of spiked samples of liver should be prepared. Hence, one laboratory will prepare it and analyse them 20 times and will send samples to a second laboratory to confirm.

  The concentrations used for spiking with the compounds will be at two levels: one around limit of quantification (low limits) and one around the middle range of biomonitoring (excluding concentrations related to poisoning).

  In addition, Andreia Freitas and Elisabeth Sharp said that they will contact EU reference laboratories and FAPAS to check feasibility about proficiency testing and/or CRM (after SETAC) Commercial labs (EU) or EU certified laboratories to organize proficiency testing (such as Federal Office of Consumer Protection and Food Safety (Germany) and University of Almeria, Spain)

- Control standards: A mix of certified standards of the compounds of interest will be sent to the participant laboratories in order to check their own standards and correct if necessary. The stability of standards should be checked.
- Blank matrix samples will also be sent to the laboratories.
- Information for the laboratories: The laboratories will receive the samples to analyse (2 aliquots of 5 g) together with an information letter including data about the range of concentrations of rodenticides used to spike the samples. Laboratories will be suggested to run 3 replicates.
- Report template: A template form to report the QA/QC criteria and method used for the analyses will also be provided to study comparability of results. The results should be reported as concentrations of each compound and for each replicate, and mean and standard deviation.
- Other issues: to send out invitations to participate to laboratories (Pilar Gómez-Ramírez will do it), to code each laboratory with a number so that the presentation of results is anonymous, to check if the costs of transport of samples can be paid by each participant laboratory. Pilar Gómez-Ramírez was chosen as the contact person to core group.
- Short term scientific missions (STSM): The preparation of samples, report and discussion of analyses results may be objectives for STSMs

Funding aspects:

Since there is no specific funding for the proof of concept, participant laboratories would work voluntarily. However, funding will be sought from relevant institutionsm and this is an issue of public health interest.

Antonio García-Fernández suggested that cross-lab comparison could be carried out by his research group within a new research project.

WORKSHOP 4th SESSION: FORENSIC DIAGNOSIS IN AR SECONDARY POISONING (Antonio García-Fernández, Rafael Mateo, Philippe Berny)

The study of anticoagulant rodenticides exposure in wildlife can be limited to measuring levels in tissues (liver) of animals found dead or admitted in rehabilitation centres. In case of finding residues of these compounds, there is a great uncertainty about the influence of AR exposure in the cause of death, especially when there are not signs of intoxication (i.e. haemorrhages in cavities). In addition, there are also uncertainties about the threshold concentrations related to intoxication in birds (ranging from 10-200 ng/g, depending on the species). Hence, there is an urgent need to deepen the knowledge in this sense, especially to be able to diagnose those cases of poisoning by anticoagulant rodenticides. For this reason, the 4th season of this workshop was devoted to discussing about several aspects.

Again, different aspects related to this subject were discussed in small groups lead by Philip Berny, Pilar Gómez and Madis Leivits.

Excluding/including criteria to select species for monitoring purposes and for secondary poisoning diagnosis

Based on some key questions in terms of monitoring and secondary poisoning (spatial distribution, temporal changes, changes in regulatory status of second generation anticoagulant rodenticides, pathway of exposure and habitat), this group selected different species of birds of prey from Europe:

- Red kite (*Milvus milvus)*
- Black kite (*Milvus migrans)*
- Barn owl (*Tyto alba*)
- Tawny owl (*Strix aluco)*
- Kestrel (*Falco tinnunculus*)
- Eagle owl (*Bubo bubo*)
- Common buzzard (*Buteo buteo)*
- Sparrowhawk (*Accipiter nisus*)
- Long-eared owl (*Otus scops)*
- Marsh Harrier (*Circus aeruginosus*)
- Golden eagle (*Aquila chrysaetos*)

How to improve obtaining information from carcasses to facilitate the presumptive diagnosis of secondary poisoning by ARs:

The group proposed that the following information should be included when a dead animal is sent to laboratory, in order to facilitate the diagnosis:

- Background information of the incident: site, habitat, large-scale eradication treatments, baits in urban areas.
- Status of conservation (decomposition) or storage (freezing) of the animal: decomposition can lead to changes in pathologic lesions
- Necropsy findings: Signs of non-clotted blood, trauma and other evidences for differential diagnosis, location of haemorrhages (See Murray 2017)

In addition, some uncertainties were discussed and pointed as necessary for a better diagnose of poisoning:

- There is scarce information on ARs degradation in carcasses during the process of body decomposition.
- There are uncertainties about the threshold concentrations related to intoxication in birds (ranging from 10-200 ng/g, depending on the species): The participants discussed between the possibility to assume the probabilistic approach of Thomas et al. 2011 or the lowest threshold level (Stone et al., 1999)
- There is a need to find sensitive biomarkers of exposure and effects: Vitamin K/epoxide in liver (live birds: plasma). There may be other genomic approaches related with clotting. In live birds, coagulation times can be measured (prothrombin time, Russell's viper venom time (Rattner et al.).

Panel of ARs to be analyzed in forensic samples

The discussion was based on the following criteria:

- Previous use
- Detection in past studies
- To confirm detection (false positive)
- The possibility to be analysed by same technique (simultaneously)

As conclusion, bromadiolone, brodifacoum, difenacoum, difethialone and flocoumafen were selected as priority compounds.

Alpha-chloralose and strychnine were considered also important rodenticides to be analysed in birds of prey, although these are not anticoagulant.

WORKSHOP 5th SESSION: SHORT TERM SCIENTIFIC MISSIONS (STSMs)

Definition of the aims for the STSMs

The main aim of the STSMs is to undertake pan-European meta-analyses of trends in exposure and poisoning of raptors and owls by SGARs.

In order to achieve this aim, several points should be considered:

- Trends in exposure but also in poisoning due to ARs should be studied
- Only peer reviewed papers should be included in the review of biomonitoring studies (following EFSA guidance)
- For poisoning, other sources of information should be considered
- Meta-analysis: how to make the database
- If possible, detailed information about location should be obtained to make a map of distribution
- It should be indicated if the studies refer to passive or active sampling
- The analytical methods should be carefully reviewed in order to confirm the comparability among studies

The results of the review may give answers to the following questions:

- Which SGARs are found in birds of prey and owls of Europe?
- What are the spatial and temporal trends of SGARs in birds of prey and owls around Europe?
- How residues vary among species and traits?

- Which are the most common lessions/clinical signs caused by SGARs in birds to diagnose intoxication?
- What information can we get about usage?
- What information do we have about sublethal effects? Are these a cause or an effect of SGARs exposure?
- What information do we have about population effects?
- What is the prevalence of exposure and poisoning?
- What are the indirect effects?
- Does exposure predispose to other causes of death?
- Do mitigation actions work?
- Can we use information of SGARs in mammals to estimate possible exposure to birds of prey?
- Examination of spatial and temporal trends in exposure and poisoning
- Can we use this information to indicate main pathways of exposure and relationship to use?
- To find key gaps

Potential hosts for STSMs

- Phillipe Berny
- Antonio J. García-Fernández
- Richard Shore
- Rafael Mateo
- Mikael Harju
- Morten Elmeros and Isabelle Fourel would ask their respective institutions about the possibility to host

Suggested criteria for selection of candidates

- Early career researchers are preferred
- Expertise in wildlife toxicology
- Published papers and conference communications (to rank)
- Quality of the cover letter
- Should have a clear idea of the scope of the STSM
- Background in statistics, GIS and database management
- Candidates should stay all longer

Dates to carry out the STSM

Minimum from June 2019- latest January 2020 (could be until April 2020)

Expected deliverables from the STSM

- Report checked by host and WG leaders
- Open access publications
- Picture gallery for ARs postmortem diagnosis to post at ERBFacility website
- Short protocol for tissue sampling at website
- Database
- Maps of distribution

Dissemination of the outputs

- Send results to authorities (EFSA and ECHA, national member states)
- Probably include the results at NORMAN Database
- Summary at ERBFacility website

- Conferences
- JRC database

Attendees and acknowledgements

The workshop in Madrid was attended by 23 participants from 9 countries (Denmark, Estonia, Finland, France, Germany, Hungary, Italy, Norway, Portugal, Slovenia, Spain, United Kingdom), mostly invited as they are involved in projects and studies on anticoagulant rodenticides.

Special thanks to local organiser Rafael Mateo and the National Museum of Natural History in Madrid - Centro Superior de Investigaciones Científicas, Spain, for logistical support. Development of the scientific programme was led by Antonio J. García-Fernández (Lead WG2), Richard Shore, (Lead WG1), Philippe Berny (WG2) and Rafael Mateo (WG2). Also thanks to Madis Leivits and Pilar Gómez-Ramírez for leading discussions and the participants for sharing their time and knowledge to contribute to make a productive workshop (Andreia Freitas, Arianna Aradis, Elizabeth Sharp, Francisco Soler-Rodríguez, Gabriella Leighton, Irene Valverde Domínguez, Isabelle Fourel, Janos Deri, Laura Monclús Anglada, Linda Rusalepp, Mikael Harju, Morten Elmeros, Octavio Pérez Luzardo, Oliver Krone, Pablo Sánchez Virosta, Silvia Espín, Zoran Žlabravec)

Appendix 1 – Workshop Programme

Wednesday 24th April	**MUSEO NACIONAL DE CIENCIAS NATURALES – MADRID (CSIC)**
14.00-14.30	**REGISTRATION**
14.30-15.30 **1st SESSION**	**INTRODUCTORY PRESENTATION AND PLAN FOR THE WORKSHOP** (Richard Shore, Antonio García-Fernández, Rafael Mateo)
15.30-16.30 **2nd SESSION**	**RODENTICIDES FOR PROOF OF CONCEPT** (Rafael Mateo)
	Outputs on ARs from Stirling Meeting (Richard)
	ARs of major concern for the agencies (EFSA, ECHA, ….) (Philippe Berny)
16.30-17.00	**COFFE BREAK**
17.00-18.30 **2nd SESSION**	Criteria for selection of ARs in the Proof of Concept: analytical, commercial, scientific, regulatory criteria must be discussed for each compound (Antonio and Rafa)
	Think the number and the specific compounds to be proposed for the proof of concept. Justification on each compound selected must be stated. (Rafa and Antonio)

Thursday 25th April	**MUSEO NACIONAL DE CIENCIAS NATURALES – MADRID (CSIC)**
09.00-11.00 **3rd SESSION**	**INTERLABORATORY COMPARISON - QC:** (Pilar Gómez, Antonio, Rafa, Richard)
	European labs analyzing anticoagulant rodenticides in raptors (or wildlife)
	Quality Control criteria in labs analysing ARs to probe an interlaboratory comparison
	Comparability of the analytical methods to be used in the proof of concept. How to interpret results already obtained with new proposed methods.
	Think about how to do an interlaboratory comparison among selected labs
	Think about the possibility to obtain financial support for interlaboratory comparison
	Think about how to make the proposals as inclusive as possible among labs. Think about how to get the labs to pledge support
11.00-11.30	**COFFEE BREAK**
11.30-13.30 **3rd SESSION**	**INTERLABORATORY COMPARISON - QC:** Mechanics of delivery - who will make it happen? How to coordinate work/samples/analyses by lab? Linked STSMs? Timescales for delivery?
13.30-14.30	**LUNCH**
14.30-16.30 **4th SESSION**	**FORENSIC DIAGNOSIS IN AR SECONDARY POISONING** (Antonio, Rafa, Philippe)
	Think about excluding/including criteria to select species for monitoring purposes and for secondary poisoning diagnosis
	Think about how to improve obtaining information from carcasses to facilitate the presumptive diagnosis of secondary poisoning by ARs
	Think about the panel of ARs to be analysed in forensic samples

	What about other no anticoagulant rodenticides? Are they a concern to be considered in raptors?
16.30-17.00	**COFFE BREAK**
17.00-19.00	**Forensic diagnosis in AR secondary poisonings** (continuation of the 4th s.)
Friday 26 April	**MUSEO NACIONAL DE CIENCIAS NATURALES – MADRID (CSIC)**
08.30-10.30 **5th SESSION**	**SHORT TERM SCIENTIFIC MISSIONS (STSMs)**
	Definition of the aims for the STSMs
	Discussion and propose of potential hosts for the STSMs
10.30-11.00	**COFFE BREAK**
11.00-12.00 **5th SESSION**	Criteria required for the potential candidates for the STSMs
	Dates to carry out the STSMs
	Deliverables expected with the STSMs

Appendix 2 – List of documents

- 1st session: