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Commentary

Discussion paper on 'Comparing the agreement of a commercial cortisol kit with a biologically validated assay in evaluating faecal cortisol metabolite levels in koala joeys'

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This Discussion paper is in response to the article by Pahuja and Narayan (2023), which compared two cortisol enzyme immunoassays (EIAs), the Arbor Assay cortisol kit and the validated R4866 cortisol assay. Our concerns relate to the experimental design and arguments in the introduction and discussion used to suggest the suitability of the cortisol EIA kit. We analyse some of the statements and respond by presenting information based on current scientific knowledge.

The koala was declared endangered in 2022 in Queensland, New South Wales and the Australian Capital Territory. Establishing correct parameters to assess stress in this species is of paramount importance. In 2013 cortisol EIAs for measuring faecal cortisol metabolites (FCMs) in koalas were validated using an ACTH challenge (Davies et al., 2013; Narayan et al., 2013). Because cortisol is heavily metabolised by the liver and bacterial enzymes in the intestine, no cortisol is excreted via the faeces (Palme et al., 2005). Still, cortisol EIAs can detect some FCMs due to cross-reactions. However, studies assessing stress in koalas using cortisol EIAs showed contradictory outcomes. Hence, the need for more specific EIAs (Fanson et al., 2017).

Santamaria et al. (2021a) identified the cortisol metabolites in faeces of koalas using liquid chromatography-mass spectrometry (LC-MS) and evaluated several EIAs. They found that the main FCM is tetrahydrocortisol (THF), best detected by a tetrahydrocorticosterone EIA (aka 50c) and that a cortisol EIA could not detect THF. Our research then determined the seasonal baseline FCM values in male and female koalas (Santamaria et al., 2021b). Again, 50c could detect differences, while the cortisol EIA did not (Santamaria et al., 2021b). Furthermore, Santamaria et al. (2023), studying the effect of illness, injury and hospitalisation on stress in wild koalas, showed that 50c EIA yields reliable results in detecting stress, while the cortisol EIA does not. Moreover, cortisol EIAs (including commercial ones) cross-react with exogenous glucocorticoids (GCs) administered as treatment during rehabilitation, which renders these EIAs unsuitable for determining stress in animals receiving this treatment, while 50c does not react with these GCs (Santamaria et al., 2023). In their paper, Pahuja and Narayan (2023) do not correctly acknowledge recent research by Santamaria et al. (2021a, 2021b) instead, this, as well as other studies, have been misinterpreted. Examples are presented below.

The authors refer to the Arbor Assay cortisol kit as 'new' and 'modern' and consider the R4866 as 'traditional'. However, both assays rely on exactly the same principle (competitive assays with directly labeled horseradish peroxidase). The only difference is that commercial kits are more user-friendly (and may provide more rapid results), but with the drawback (besides higher costs) that even with these perceived 'easy-to-use' kits, a very high level of experience and skill is required to enable reliable interpretation of results. In fact, both cortisol EIAs have similar cross-reactivities (cortisol is always set at 100%), because commercial cortisol EIA kits contain a similarly produced antibody. Although companies usually hide their details, both immunogens are most likely coupled at position C3 (cortisol-3-CMO:BSA). Most importantly, none of them can detect THF. Furthermore, explanations in the discussion mention cortisol binding globulins in the commercial assay, but there are no cortisol binding proteins in the faeces (only in the blood), and why should other faecal proteins bind to the antibodies designed to capture the small molecule cortisol?

The authors also consider the EIA used in our research as 'traditional'. However, it is in all aspects more modern and advanced, because it was designed as a so-called group-specific assay and utilizes biotinylated labels, which have several advantages. In many species, such group-specific EIAs have proven superior over cortisol assays (no matter whether home-bred or commercial). This is now widely recognised by most experts in the field (Palme, 2019).

The authors argue that an ACTH challenge in joeys is unethical, but do not consider alternatives such as biological validations that can be performed using naturally-occurring (or even necessary: e.g., vet exam) stressors (Santamaria et al., 2023) to demonstrate that the utilised EIA can detect the expected signal (increased FCM levels) in serial samples

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(Palme, 2019).

The authors state that the sampled animals represented a suite of clinical conditions, ranging from healthy to injured to diseased individuals, or that they were suited for a biological validation. However, there were only four koalas and a sample size of $N = 34$, which is extremely low, especially for a correlation study. Moreover, paradoxically, the authors also state that koalas were all healthy when the faeces were collected. However, the outcome shown in Table 1, tells a different story. In addition, no details are provided on the length of hospitalisation, any medications administered, or other potential stressors incurred during their rehabilitation or, indeed, when exactly the scats were collected. It also remains unclear what further 'laboratory validation' needs to be performed. Instead, a sound physiological/biological validation would be mandatory (Palme, 2019). Thus, no sound conclusions can be drawn about the suitability of the commercial EIA in detecting stress in koala joeys.

In the introduction, the authors mention a prolonged gut transit time of eucalypt fibre in koalas and relate this to the release of GCs. However, the citation of Keay et al. (2006) is a secondary source and did not measure koalas gut transit time. Further, the statement 'Due to this prolonged gut transit time (~ 9 days) faecal based hormone monitoring technique is highly suitable for koalas' is not supported by the literature. In fact, the cited paper (Narayan and Vanderneut, 2019) refers to an earlier study (Narayan et al., 2013), which analysed the delay of cortisol excretion from plasma to faeces and did not investigate the link between digestion and the gut transit time, which has not been determined in koalas. Moreover, Narayan et al. (2013) showed that koala FCMs peaked one or two days after ACTH injection and returned to baseline values after around 3–4 days. Here, the authors fail to mention Santamaria et al. (2021a) where frequent serial samples revealed an even shorter (around 11 h) lag time from blood to faeces in koalas. Knowledge of this delay is essential for non-invasive monitoring to understand which physiological situation before the sample collection is reflected by the measured FCMs.

In conclusion, whether the two assays are in agreement is not the point. Correlational studies are unsuited as validation (Palme, 2019) and researchers should aim at finding, and using, the best suited (sensitive in biological terms) assays, which will help to evaluate even minor stressors. Thus, the experimental design used to demonstrate the suitability of a commercial cortisol assay kit for assessing stress in koalas is flawed. On the contrary, we reiterate that cortisol assays are less sensitive for measuring stress in koalas, because they do not cross-react with the main FCM. Our concern is that this paper seeks to provide justification for the use of commercial cortisol EIAs to detect stress in this species. Unfortunately, the senior author's recent post in The Conversation (Narayan, 2022), underlines this. However, researchers need to be aware that using non-validated (thus possibly invalid) assays, might be more harmful than beneficial to the target species.

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