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Analytical validation of an Enzyme Immunoassay for the measurement of urinary oxytocin in dogs and wolves

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ABSTRACT

Assessing changes in oxytocin (OT) levels in response to a variety of social stimuli has become of major interest in the field of behavioral endocrinology. OT is involved in the regulation of various aspects of social behavior such as tolerance, and the formation and maintenance of social bonds but also the regulation of stress. All of these aspects have been identified as potential targets of selection during the domestication process. Therefore, comparing the role of the oxytocinergic system in various aspects of dog and wolf social behavior, might help to understand whether this system was involved in the domestication process.

Studies assessing OT levels in dogs and wolves have used invasively collected plasma and serum samples and non-invasively collected urine samples. However, when using an assay system on a new species a careful and complete validation of the method is of crucial importance, and to date no proper validation, to assess urinary OT levels in dogs and wolves, has been reported. We therefore conducted an analytical validation of an Enzyme Immunoassay (EIA) for the measurement of OT in urine of dogs and wolves, using a commercially available EIA. Stability tests revealed that OT levels degrade over time when stored at 4 °C, but are little affected by repeated thawing. In addition, our results indicate that the variance in OT levels is slightly lower when phosphoric acid is added following collection to prevent OT degradation. Long term storage tests revealed that urinary OT levels are least variable when stored as extracts in ethanol at −20 °C, rather than as unextracted urine samples. Validation results were acceptable with regard to parallelism, but values for accuracy and extraction efficiency were not meeting the standard criteria usually applied to steroid EIAs, especially when assessed for the lower range of the assay. The results of this study highlight the importance of an analytical assay validation, since even if validation parameters are not optimal, if published, they allow readers to estimate the relevance of studies using the validated method.

1. Introduction

Over the last decades the neuropeptide hormone oxytocin (OT) has become one of the most studied hormones in relation to social behavior and social bonding. Originally known to be involved in processes of parturition and milk let down (Russell and Leng, 1998), the oxytocinergic system has been intensively studied in monogamous prairie voles and found to be related to e.g., mate preferences (Carter et al., 1992; Williams et al., 1994), alloparental care (Bales et al., 2004) and maternal behavior (Olazábal and Young, 2006). Furthermore, OT has been shown to play a pivotal role in the formation and maintenance of social bonds (reviewed in e.g., Crockford et al., 2014; Ziegler and Crockford, 2017), to be involved in male and female sexual behavior (reviewed in e.g., Park and Rissman, 2011), trust (Kosfeld et al., 2005), food sharing (Wittig et al., 2014), between group aggression and within group cooperation (De Dreu, 2012; Samuni et al., 2016) and the regulation of stress (reviewed in e.g., Olff et al., 2013; Buttner, 2016; Brown et al., 2016). Recently researchers have started to investigate the role of OT in the human-dog bond (Handlin et al., 2011; Mitsui et al., 2011; Nagasawa et al., 2015; Odendaal and Meintjes, 2003; Rehn et al., 2014) and the oxytocinergic system has been suggested to be one of the key factors involved in the domestication process of dogs (Nagasawa et al., 2015).

OT is a neuropeptide hormone, which is produced in the

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hypothalamus and released by the posterior pituitary into the bloodstream or transported via axons into the brain, but also reported to be synthesized in various peripheral tissues (Gimpl and Fahrenholz, 2001). Numerous studies investigated the role of OT by assessing it centrally, i.e., measuring OT in cerebrospinal fluid (Amico et al., 1990; Carson et al., 2015; Devarajan and Rusak, 2004; Winslow et al., 2003). However, measuring central OT levels is not feasible in most studies due to its heavily invasive nature and thus the assessment of hormone levels using peripheral measurements, i.e., in blood (Carson et al., 2015; Feldman et al., 2007; Morris et al., 1980) and urine samples (Crockford et al., 2013; Finkenwirth et al., 2015, 2016; Reyes et al., 2014) has become increasingly common. The reliable assessment of OT in urine samples is of special interest, for two main reasons: first, in many mammalian species urine samples can be assessed non-invasively without restraining animals to collect blood samples; and second, in contrast to blood, urinary hormone levels provide an integrated measure thereby reflecting levels of the hormone over a certain period of time. Unfortunately, the majority of publications do not present detailed data on the analytical validation for the measurements of peripheral OT levels. Using methods without establishing their validity beforehand might hinder the correct biological interpretation of results. Indeed, while a number of studies refer to previously reported methods, they still implement significant methodological changes, such as using a different assay system (with a different antibody with different cross-reactivities), applying the method to another species or matrix, or omitting indispensable extraction steps. This has often led to non-replicable findings and erroneously high OT levels (reviewed e.g., in Leng and Sabatier, 2016; McCullough et al., 2013; Ziegler, 2018). So far, studies investigating the effect of applied methodologies have mostly concentrated on plasma samples and reported non-matching OT levels when compared across assays and with or without the use of extraction (Robinson et al., 2014; Szeto et al., 2011; for saliva see: MacLean et al., 2017; for a comparison of different extraction methods and assay systems, using human plasma see Brandtzaeg et al., 2016).

Given that the validity of peripheral measures of OT in blood, saliva and urine is still a hotly debated topic (Horvat-Gordon et al., 2005; Leng and Sabatier, 2016; McCullough et al., 2013; Szeto et al., 2011), a careful analytical validation before use in research, is of crucial importance. In general, an analytical validation must comprise a number of steps. First, various assay parameters, such as parallelism to investigate the presence of matrix effects, i.e., substances that could interfere with the assay system, need to be evaluated. Antibody specificity of the EIA can be assessed via immunograms. Furthermore, tests to determine the efficiency of the extraction method and the reliability of the assay, must be conducted. Second, various sample parameters, such as the stability of the hormone of interest and its degradation across various storage methods should be investigated. Given the short half-life of OT in blood (3–6 min in humans; Fabian et al., 1969), assessing the stability of urinary OT is of major interest. Finally, exploring additional practicalities, such as accuracy of repeated measurements of the same sample or the effect of concentrating a sample (needed when hormone levels are very low), are important before conducting studies investigating hormone and behavior interactions.

The aim of the present study was to provide a thorough analytical validation to assess OT levels in dog and wolf urine samples, thereby potentially establishing a valid tool (with practical guidelines) for use in future studies relating to OT-behavior interactions. To this aim, we first conducted a test for parallelism to assess the presence of matrix effects, determined extraction efficiency and assay accuracy and identified immunoreactivity patterns in dog and wolf urine samples to test the specificity of the antibody used in the Enzyme Immunoassay (EIA) used in this study. Furthermore, we investigated the stability of urinary OT during short term storage at 4 °C and room temperature, during long term storage as untreated samples at −20 °C or −80 °C or as evaporated extracts stored in ethanol. In addition, we assessed whether OT degrades over repeated freeze-thaw cycles. We explored the effect of repeatedly extracting and measuring urinary OT from the same or different aliquots. Finally, we assessed whether concentrating samples, often necessary when hormones are only present in low concentrations, affects OT levels.

2. Methods

2.1. Subjects

Urine samples of 13 pet dogs (7 females, 6 males) were collected at the Max-Planck-Institute for Evolutionary Anthropology (MPI EVA) in Leipzig, Germany. Urine samples of 3 pet dogs (2 females, 1 male) and urine samples of 9 wolves (4 females, 5 males) were collected at the Wolf Science Center (WSC) in Ernstbrunn, Austria. All individuals were in good health status at the time of sample collection.

Ethical approval was obtained from the ethical commission of the University of Veterinary Medicine, Vienna (Approval number: ETK05/03/2017) for wolves, and from the ethical commission of the Max Planck Society (Approval number: 2017_07), for dogs.

2.2. Sample collection

All dog urine samples were collected when the dogs were taken for a walk by their owner in front of the MPI EVA. Voluntarily voided urine samples were collected in plastic trays (Carl Roth, CEN 7.1) and directly brought to the Endocrinology Lab at the MPI EVA (within 5 min), where 1 ml aliquots were created immediately. To avoid degradation of oxytocin, urine samples were acidified by adding 100 µl of a 0.1% phosphoric acid per 1 ml of urine (Crockford et al., 2013; Samuni et al., 2016) and frozen directly at −20 °C.

At the WSC, dog urine samples were collected by the owners in plastic trays. Immediately following collection, 100 µl of a 0.1% phosphoric acid per 1 ml of urine were added, and samples were stored at −20 °C until transport on dry ice to the MPI EVA.

Most wolf urine samples were also collected during leashed walks with their trainers. All wolves at the WSC are used to be taken on leashed walks, and tolerant of humans kneeling behind them to collect the samples in a small tray (as with the dogs). In cases when urine could not be collected directly using this method, urine was pipetted from leaves or from the ground (the latter in some cases when animals urinated in an indoor area), or urine was pipetted from plastic bowls which were placed in different locations in an outdoor enclosure. Urine samples were only used when contamination with other urine samples could be excluded. All samples were directly stored on icepacks for transport to the freezer (maximum of 60 min) and then directly aliquoted (as dog urine samples above) and stored at −20 °C until transported on dry ice to the MPI EVA.

2.3. Sample preparation and urinary oxytocin measurements

All laboratory analyses were conducted in the Endocrinology laboratory at the MPI EVA. Until used for validation, all urine samples were stored at −20 °C or at −80 °C when used for storage and stability tests (see details in respective section). To assess immunoreactive urinary oxytocin metabolites (iuOTM), preceding extraction, urine samples were thawed, but kept at 4 °C on wet ice or cold iso-racks (Eppendorf) until loaded on SPE (solid phase extraction) cartridges. All samples underwent a solid phase extraction (SPE) following Crockford et al. (2013), with some minor modifications. In detail, when thawed, samples were gently vortexed for 10 s and centrifuged for one minute at 1500 rpm. SPE cartridges (Chromabond HR-X, 1 ml, 30 mg) were conditioned with 1 ml methanol (100%, HPLC grade) and 1 ml HPLC water. Following that, 0.1% trifluoroacetic acid (TFA) and the urine sample were loaded onto the cartridge. The volume of urine and TFA loaded onto the cartridge varied across steps of the analytical validation. Detailed information on extracted volumes are therefore given in the respective parts of the methods section. Cartridges were washed five times with 1 ml 10% (vol/vol) acetonitrile (ACN) containing 1% TFA in water, sucked dry with a vacuum pump and samples were eluted with 1 ml 80% (vol/vol) ACN. Eluates were evaporated until complete dryness at 50 °C using a gentle stream of compressed air and reconstituted in 300 µl 100% ethanol. Following another gentle vortexing for 10 s samples were kept at 4 °C in a fridge for a one-hour incubation period. Samples were evaporated again and finally reconstituted in 250 µl of OT assay buffer (supplied along with the commercial assay kit; Enzo Life Sciences Assay designs, Cat. No. 901-153A-0001), gently vortexed for 10 s, centrifuged for one minute at 10,000 rpm and assayed according to the suppliers' instructions. Per well, 100 µl of the extract were brought to the assay. All samples were measured in duplicates and repeated or excluded when OD values differed for more than 10%. Inter-assay coefficients of variance of OT levels were 15.4% for a high concentrated OT standard and 15.9% for a low concentrated OT standard (n = 15). Intra-assay coefficient of variance of OT levels was 11.3% (n = 31) and was determined by calculating the average variability across duplicates of 31 samples measured in a single assay.

To assess the impact of inter-individual differences, some parts of the validation were conducted using individual urine samples rather than pooled urine samples. For those, we measured creatinine (crea) in the urine samples to compensate for volume and concentration of the urine (Bahr et al., 2000; Jaffe, 1886). All iuOTM levels were expressed as pg/mg creatinine.

2.4. Analytical validation

2.4.1. Parallelism

We investigated matrix effects that could potentially interfere with the assay system by testing for parallelism. For each species a 450 µl pool sample, each consisting of three individual samples, was spiked with 50 µl of a 4000 pg/ml OT standard (supplied by Enzo Life Sciences, Assay designs) and diluted serially. As the concentration of iuOTM in these two species can be low, we spiked the pool samples with an OT standard to get a meaningful dilution curve that allows the assessment of matrix effects at concentrations where samples are routinely measured (see also Schaebs et al. (2016)).

2.4.2. Extraction efficiency

We created 5 pools of dog and 5 pools of wolf urine samples. Before extraction 237.5 µl of each pooled sample were spiked with 12.5 µl of three different concentrations of an OT standard (high = 4000 pg/ml; medium = 2000 pg/ml; low = 1000 pg/ml). We used the values from the spiked and unspiked samples to calculate percent recovery for extraction efficiency and assay accuracy following the formula given in Behringer et al. (2012):

$$Rec\,(\%) = \frac{(Ssp - a \times Sm)}{(b \times Std)} \times 100$$

with Ssp being the measured concentration of the spiked sample, Sm being the measured concentration of the unspiked sample, Std being the concentration of the standard, a being the amount of sample and b being the amount of standard.

2.4.3. Assay accuracy

We created 5 pools of dog and 5 pools of wolf urine samples. Following extraction, 237.5 µl of pooled urine extracts were spiked with 12.5 µl of three different concentrations of an OT standard (high = 4000 pg/ml; medium = 2000 pg/ml; low = 1000 pg/ml) and spiked and unspiked samples were analyzed for iuOTM concentration. The calculation of the assay accuracy was performed with the formula given above.

2.4.4. Immunograms

We investigated patterns of immunoreactivity within dog and wolf urine samples by creating immunograms (a graph depicting areas of immunoreactivity obtained by collecting and assaying fractions of standard or sample) of dog and wolf urine samples. To do so, we prepared one pooled urine sample consisting of samples from five dogs and a second pooled urine sample consisting of five wolf urine samples. In addition, we prepared a 2000 pg/ml OT standard (supplied with the assay system Enzo Life Sciences, Assay designs) and extracted the OT standard and the two pool samples as described above and reconstituted each in 150 µl 30% ACN.

We ran 100 µl of the extracts of the dog and wolf pooled urine samples and OT standard over a Waters Alliance 2695 HPLC equipped with a Gemini C18 column (Phenomenex, Torrance, CA, USA) with a flow rate of 0.2 ml/min using a gradient of eluent A (5% acetonitrile with 0.1% formic acid) and eluent B (95% acetonitrile with 0.1% formic acid). We collected 12 fractions of 600 µl every 3 min with a Waters Fraction Collector 3 (Waters, Milford, MA, USA). Fractions were lyophilized overnight and kept frozen at −20 °C until they were reconstituted in 250 µl of assay buffer for further analysis with the Immunoassay.

We determined the immunoreactivity (IR) in each fraction (obtained from the HPLC) through measurements on the EIA. We first determined in which fractions of the OT standard IR was found. The sum of IR found across all fractions was labeled total IR. We then determined for each urine pool the amount of IR found in the fractions in which IR was as well present for the OT standard. The sum of IR in these fractions was labeled explained IR, that is IR that overlapped with the standard and therefore most probably stems from OT or one of its metabolites. We then determined for each urine pool the amount of IR found in fractions where no IR was found in the OT standard. The sum of IR in these fractions was labeled unexplained IR, that is IR that did not overlap with the standard and therefore most probably stems from substances not coming out of the OT metabolism. We then calculated the proportion of explained and unexplained IR according to the following formula:

$$proprotion\ of\ unexplained\ IR\,(\%) = \frac{total\ IR\ -\ sum\ of\ IR\ found\ in\ OT\ Std}{total\ IR} * 100$$

$$proprotion\ of\ explained\ IR\,(\%) = 100\ -\ proportion\ of\ unexplained\ IR$$

2.4.5. Repeatability

We aliquoted a subset of 5 dog urine samples at the time of collection into subsamples. We tested repeatability within and across aliquots by extracting and measuring each sample three times out of the same aliquot and out of three different aliquots. We then calculated the average iuOTM concentration, standard deviation and the coefficient of variance.

2.4.6. Storage & stability

To assess degradation patterns of iuOTM levels three different sets of tests were conducted. First, to investigate the effect of short term storage on samples at 4 °C or room temperature (RT), we prepared 2 dog urine sample pools and created aliquots to be frozen immediately after collection and 1 h, 3 h, 6h and 24 h following collection. As previous studies added phosphoric acid to their urine samples to inhibit OT degradation (Crockford et al., 2013; Samuni et al., 2016), we assessed the effect of phosphoric acid by testing each condition with having phosphoric acid (PA) added to the aliquots or without adding acid.

Second, to test for the impact of repeated freezing and thawing on iuOTM levels, we subjected 2 dog urine sample pools to three freezing/thawing cycles. Again, this test was conducted with and without added PA. The effect of thawing was also tested using wolf urine sample pools (here, only urine with PA added was used).

Third, to investigate the effect of long term storage on iuOTM levels, we stored a set of four dog and four wolf urine samples (each 2 males and 2 females) either as samples at −20 °C or at −80 °C or as extracts stored in 300 µl 100% EtOH for 2 weeks, 4 weeks, 12 weeks and 6 months.

2.4.7. Effect of concentrating standard on iuOTM levels

Despite acceptable values of parallelism, variance in measured urinary OT levels can depend on sample dilution or concentration (Amico et al., 1987). We therefore assessed whether concentrating samples during the extraction procedure might affect measured iuOTM levels. We measured three dog urine samples at two different concentrations, either not concentrated or concentrated four times. For assessment of urinary OT levels in samples that were not concentrated, 250 µl of the samples were loaded onto the cartridge and, following extraction, reconstituted in 250 µl assay buffer. To assess urinary OT levels in the same samples after being concentrated four times, 1000 µl of dog urine sample were loaded onto the cartridge and reconstituted in 250 µl assay buffer.

To investigate whether discrepancies in results were due to matrix effects in the urine samples, we repeated this test using an OT standard dissolved in assay buffer. For this, aliquots of a 400 pg/ml OT standard were extracted and reconstituted in 1000, 500 and 250 µl of assay buffer. Measured values were corrected for the concentration factor and compared.

2.4.8. Additional methodological tests

We tested two more additional factors that, during extraction, could have an effect on resulting variance in OT levels. First, the impact of the duration of vortexing was compared, as this could have an effect on the efficiency of reconstituting substances in tubes. We extracted three different dog urinary sample pools (each three times, resulting in N = 9) and either vortexed each sample pool ten seconds or three minutes at all steps during the extraction procedure where vortexing was performed.

Second, we assessed the effect of the timing when, during the sample extraction process, TFA was added to the sample or to the cartridge, as this might affect how substances bind to the sorbent of the cartridge. Here, we extracted three different dog urinary sample pools (each three times, resulting in N = 9) and either mixed the samples with TFA preceding loading the sample onto the cartridge or we first added TFA to the cartridge and then loaded the urine sample.

2.5. Statistical analysis

All plots were created using R (version 3.3.3; R Core Team, 2017). To compare urinary OT levels when tested for the effect of duration of vortexing and for the effect of mixing the samples with TFA, we ran a Wilcoxon-signed-rank test. Parallelism was tested by fitting a linear model including the interaction between the sample type (standard curve and pool sample) and the concentration of the standard (log transformed) with the optical density being the response variable (Schaebs et al., 2016). The model was fitted in R (R Core Team, 2017) using the function lm. We inspected a qq-plot of the residuals and residuals plotted against fitted values (Field, 2009) to check for assumptions of normality and homogeneity of the residuals, which did not indicate problems. Furthermore, model stability was assessed by means of DFBeta (Field, 2009), which as well did not indicate any problems.

3. Results

Average OT levels of individual dog urine samples were 70.9 pg/mg creatinine (N = 5; SD = 28.3; range = 34.7–95.4; median = 82). No concentration corrected individual levels were assessed for wolf urine samples, because comparisons were made mainly using pooled samples.

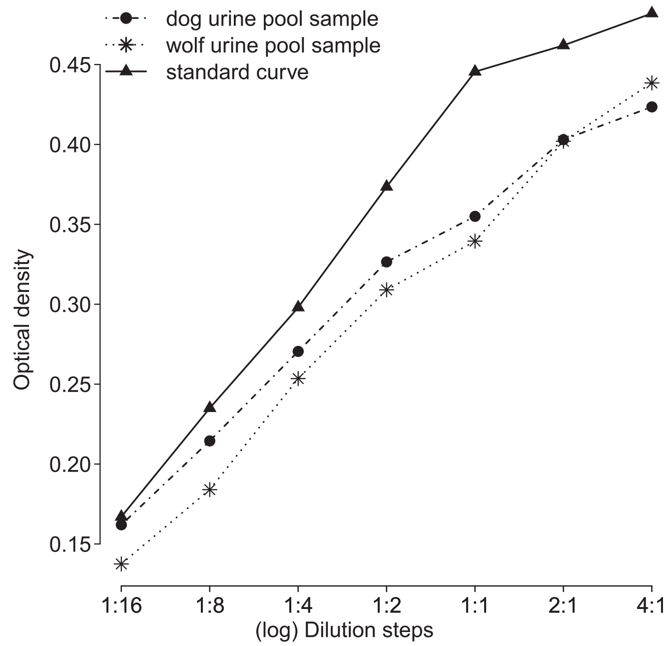

Fig. 1. Parallelism of serially diluted dog urine pool sample and wolf urine pool sample to the standard curve (dog urine: $t_{(10)} = -1.879$, P = 0.0896; wolf urine: $t_{(10)} = 0.794$, P = 0.445). Note that the x-axis is on a log scale.

3.1. Parallelism

The serially diluted sample pools of dog and wolf urine were both parallel to the standard curve (dog urine: $t_{(10)} = -1.879$, P = 0.0896; wolf urine: $t_{(10)} = 0.794$, P = 0.445), as confirmed by visual inspection (Fig. 1).

3.2. Extraction efficiency & assay accuracy

Mean extraction efficiency for spiked dog urine pool samples was 132.3% (range: 120.9%–154.4%, SD = 15.2, n = 4) when spiked with a high, 156.6% (range: 136.5%–174.8%, SD = 14.2, n = 5) when spiked with a medium, and 124.9% (range: 67.7%–197.4%, SD = 46.9, n = 5) when spiked with a low concentrated OT standard.

Mean extraction efficiency for spiked wolf urine pool samples was 131.9% (range: 124.4%–144.6%, SD = 10.9, n = 3) when spiked with a high, 137.16% (range: 115.8%–157.4%, SD = 20.8, n = 3) when spiked with a medium, and 108.5% (range: 62.9%–139.8%, SD = 37.6, n = 4) when spiked with a low concentrated OT standard.

The average assay accuracy for spiked dog urine extracts was 145.3% (range: 128.7%–170.3%, SD = 16.3, n = 5) when spiked with a high, 164.3% (range: 143.6%–173.1%, SD = 12.1, n = 5) when spiked with a medium, and 155.6% (range: 105.6%–246.4%, SD = 57.7, n = 5) when spiked with a low concentrated OT standard.

The average assay accuracy for spiked wolf urine extracts was 98.5% (range: 68.7%–117.4%, SD = 26.07, n = 3) when spiked with a high, 113.9% (range: 82.9%–162.9%, SD = 42.9, n = 3) when spiked with a medium, and 144.32% (range: 65.9%–184.7%, SD = 54.2, n = 4) when spiked with a low concentrated OT standard.

3.3. Immunograms

The immunogram of the extracted OT standard revealed immunoreactivity in fractions 2, 3, 6 and 7 (accounting for 24.1%, 57.2%, 18.1% and 0.7% of the total immunoreactivity, respectively) (Fig. 2a and b).

In extracted dog urine, we found immunoreactivity in fractions 2, 3, 6, 7 and 8 (accounting for 8.1%, 23.6% 19.6%, 28.9% and 19.9% of the

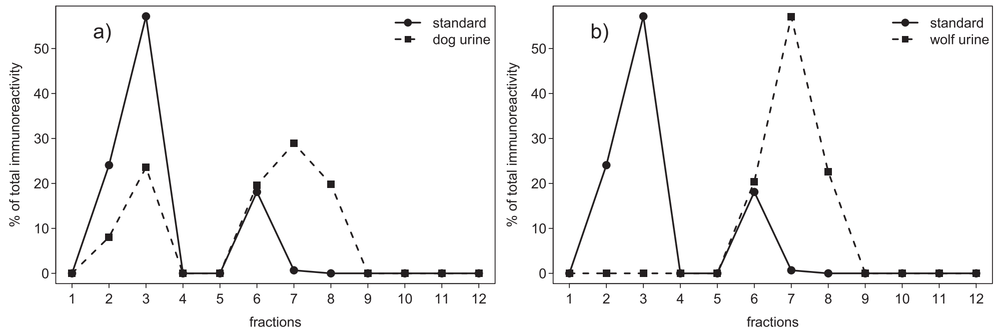

Fig. 2. Immunoreactivity in (a) extracted OT standard and extracted dog urine; and (b) extracted OT standard and extracted wolf urine.

total immunoreactivity, respectively) (Fig. 2a). The immunogram of extracted wolf urine showed immunoreactivity in fractions 6, 7 and 8 (accounting for 20.4%, 57.0% and 22.6% of the total immunoreactivity, respectively) (Fig. 2b).

Consequently, comparing the immunoreactivity for OT standard to immunoreactivity in extracted dog and wolf urine, we found that 80.15% of the immunoreactivity in the extracted dog and 77.45% of the immunoreactivity in the extracted wolf urine, could be explained by immunoreactivity found in extracted OT standard.

3.4. Repeatability

Repeated extraction and measurement of the same sample from the same aliquot ($N_{aliquot} = 3$; $N_{individuals} = 5$) revealed an average variance in iuOTM levels of 7.46%. Repeated extraction and measurement of the same sample but from different aliquots ($N_{aliquot} = 3$; $N_{individuals} = 5$) gave an average variance of 15.36%.

3.5. Storage & stability

3.5.1. Short term storage before freezing samples

When phosphoric acid (PA) was added to the urine samples immediately following collection and samples stored at 4 °C, OT levels stayed initially constant for up to 6 h when an average decrease in iuOTM levels of 6.6% (range: −18.8% to +14.8%) was observed. After a total storage duration of 24 h, average iuOTM levels decreased by 36.7% (range: 31.6%–41.7%) (Fig. 3). If no PA was added to the freshly collected urine samples, average iuOTM levels showed an initial increase of +16.0% (range: +12.9% to +19.1%) when stored at 4 °C for 1 h. Thereafter, average iuOTM levels declined by 8.4. % (range: −0.3% to −16.6%) when stored for up to 6 h as compared to the initial average iuOTM concentration. When stored for up to 24 h, average iuOTM levels increased again to +12.9% (range: +10.8%–15.0%) (Fig. 3). IuOTM levels in samples stored at room temperature appeared to be less stable (see SI for details).

3.5.2. Exposure of dog pooled urine samples to repeated thawing

IuOTM levels in pooled dog urine samples changed only slightly over the course of three freeze-thaw cycles, regardless of whether PA was added or not (Fig. 4). In pooled dog urine samples, iuOTM levels varied between +17.9% and −13.9% (average: +1.7%) when PA was added to the samples and between +13.8% and −17.1% when no PA was added (average: −2.8%) (Fig. 4). In wolves, iuOTM levels first decreased, when thawed twice and then increased above control levels after three repeated freeze-thaw cycles (see SI for details).

3.5.3. Long term storage

In general, dog iuOTM levels increased with increasing duration of storage, independent of storage method. After 6 months, samples that were stored as samples at −20 °C increased on average by 71.8% (range: +13.9% to +129.9%), and by 81.4% (range: +53.9% to

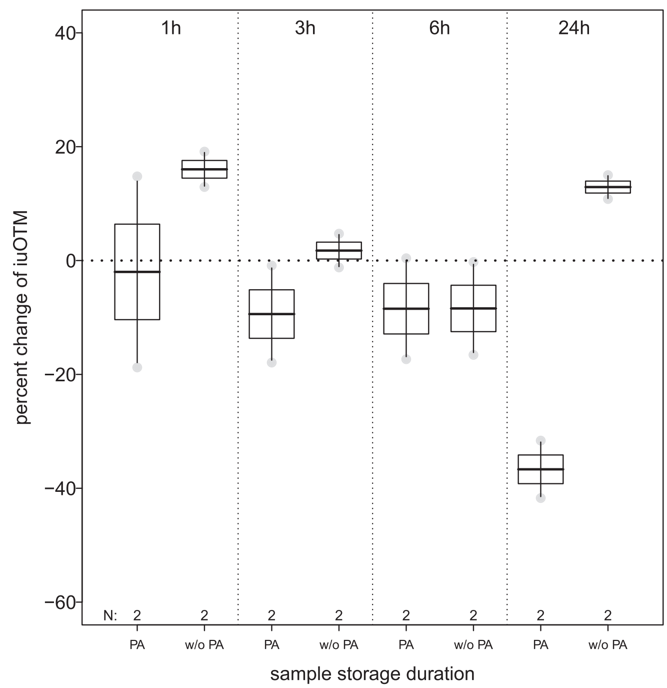

Fig. 3. Percent change in immunoreactive urinary oxytocin metabolite (iuOTM) levels of dog urine pool samples (N = 2) stored at 4 °C for 1 h, 3 h, 6 h and 24 h; separated by samples to which phosphoric acid (PA) was added or not (w/o PA). The horizontal line indicates the 100% control iuOTM levels, for pooled samples that have been frozen immediately following collection.

+133.8%) when being stored as samples at −80 °C. Samples that were extracted preceding long term storage and then stored as ethanol extract at −20 °C increased on average by 32.1% (range: +16.2% to +48.0%) (Fig. 5) when being stored for up to six months. Similarly, wolf iuOTM levels increased with increasing duration of storage (see SI for details).

3.6. Effect of concentrating standard on iuOTM levels

Concentration of the urine samples during the extraction process led to an underestimation of OT levels by on average 43.2% (range: 25.5%–58.5%, Table 1). The same pattern was found for OT standard solutions in assay buffer. The stronger standards were concentrated during the extraction process, the more OT levels were underestimated. While a concentration factor of two led to OT levels being 24.3% lower, a concentration factor of four led to OT levels being 41.3% lower when compared to non-concentrated aliquots (Table 1).

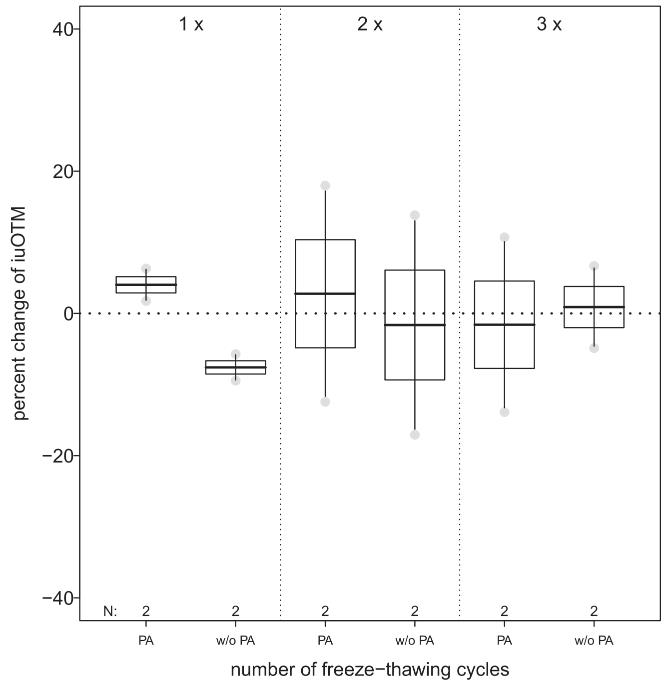

Fig. 4. Percent change of immunoreactive urinary oxytocin metabolite (iuOTM) levels in dog urine pool samples (N = 2) over the course of three freeze-thawing cycles; separated for samples to which phosphoric acid (PA) was added or not (w/o PA). The horizontal line indicates the 100% control iuOTM level, which was not exposed to thawing.

3.7. Additional methodological tests

There was no significant difference in urinary OT levels of samples vortexed for 10 s or three minutes (V = 26, p = 0.73). Furthermore, the timing of adding TFA did not have a significant effect on urinary OT levels (V = 31, p = 0.36).

4. Discussion

With this study we provide an analytical validation for the measurement of iOTM in urine samples of dogs and wolves. We validated a commercially available Enzyme Immunoassay that, according to the developer, is compatible to assess OT in human specimen (serum, plasma, saliva, breast milk, urine and cerebrospinal fluid) and that has, at least partly, been validated for the assessment of OT in urine and plasma of humans and several primate species (summarized in Ziegler (2018)). However, by validating the assay for the measurement of iOTM in urine samples (iuOTM) of dogs and wolves, we found several problems that need to be taken in consideration when assessing iuOTM levels, highlighting the importance of validating the methods for each species and matrix.

With regard to parallelism, iuOTM can be assessed reliably in these two species using the Enzyme Immunoassay provided by Enzo Life Sciences. Average levels for extraction efficiency were higher than 100% for both species and all spiking concentrations with considerably high standard deviations. A similar pattern was found for assay accuracy with particularly high standard deviations found for low spiking concentrations in dogs and for all spiking concentrations in wolves. This indicates that especially when measuring samples at lower concentrations, accuracy is far from optimal. There is a need for future studies to test alternative urine extraction methods, for example, investigating different types of solid phase cartridges, extraction solutions and evaporation methods. In addition, already published work lacking the necessary validation parameters, must be interpreted with caution, keeping in mind the mentioned difficulties here.

Furthermore, detailed immunograms suggest the presence of oxytocin or its metabolites in dog and wolf urine. However, as immunoreactivity appeared to be present in several fractions, there is evidence that other substances are caught by the assay system. For long term storage (up to 6 months), we found that iuOTM was most stable when stored as ethanol extracts at −20 °C, compared to when stored as urine sample at −20 °C or at −80 °C. Importantly, we found that changes in iuOTM levels across individual samples were variable, showing no clear pattern in the direction of change in the relation to storage duration (see Figs. SI 3 and SI 4). Adding a concentration step during the extraction appears to be leading to an underestimation of iuOTM levels. Finally, the duration of vortexing during extraction and the timing of mixing urine samples with TFA did not affect urinary OT levels.

Comparing average urinary OT levels obtained in the present study, to average OT levels of already published work puts our OT levels in about the middle of the range of what has been reported. However, most of the studies on urinary OT in dogs have been conducted using radioimmunoassays (RIAs) of a different assay provider and therefore using a different antibody, with samples being extracted but also unextracted across studies (Mitsui et al., 2011; Nagasawa et al., 2015; Romero et al., 2014). These different methodologies hinder the direct comparison of urinary OT levels across studies. The only study, to our knowledge, using the same EIA as in the present study, reports about four times lower median OT levels for dog urine (Pekkin et al., 2016). The extraction method used in the present study and the extraction method used by Pekkin et al. (2016) differed slightly and dog urine samples in the latter study were concentrated four times during extraction. These discrepancies in extraction procedures likely contribute to different OT levels measured with the same assay system since, as we

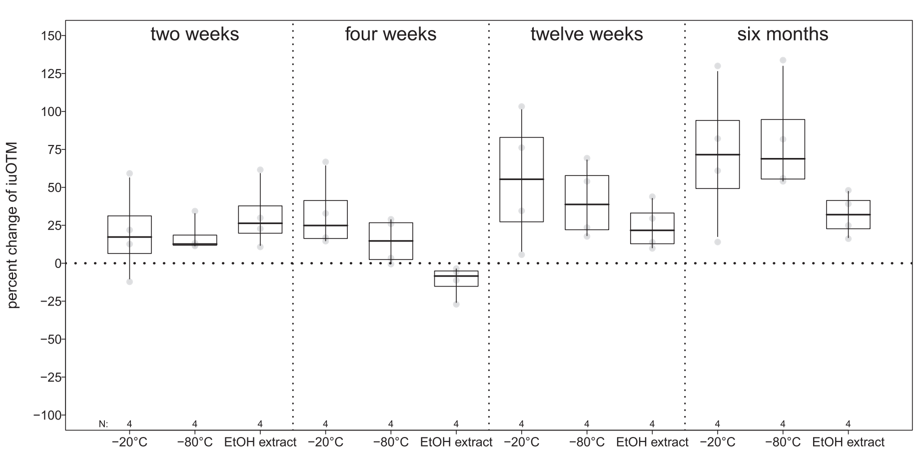

Fig. 5. Percent change in immunoreactive urinary oxytocin metabolite (iuOTM) levels of four dog urine samples after storage as sample at either −20 °C or −80 °C or as ethanol (EtOH) extract for 2 weeks (corrected for QC), 4 weeks, 12 weeks and 6 months. The horizontal line indicates the 100% control iuOTM levels (i.e, zero change).

Table 1
Effect of concentrating OT standard or urinary sample during the extraction process on OT levels.

Sample_type	Volume on cartridge (µl)	Volume reconstituted (µl)	Concentration factor	OT level measured on assay (pg/ml)	OT level corrected for concentration (pg/ml)
Sample 1	1000	250	4	399.13	99.78
Sample 1	250	250	1	184.08	184.08
Sample 2	1000	250	4	216.06	54.01
Sample 2	250	250	1	130.02	130.02
Sample 3	1000	250	4	539.12	134.78
Sample 3	250	250	1	180.78	180.78
Standard	1000	250	4	794.30	198.58
Standard	1000	500	2	512.08	256.04
Standard	1000	1000	1	338.16	338.16

showed in this study, concentrating urine samples led to an underestimation of OT levels.

4.1. Immunograms

Identifying the immunospecificity of an assay with regard to the hormone of interest is one of the major steps during a validation process to assure that the assay system at hand reliably measures the hormone of interest. This becomes especially of interest when other species than humans and other matrices than serum or plasma are used, as most of the commercially available assays were developed to measure hormones in human plasma or serum.

The immunogram of the OT standard revealed immunoreactivity to be present in several fractions, with two peaks. While one would expect one single peak for a standard, it is likely that during the extraction process functional groups are cleaved off the OT molecule. Indeed, OT is reportedly susceptible to heat and pH stress, leading to degradation of the OT molecule (Hawe et al., 2009). As long as these OT metabolites still crossreact with the antibody they create immunoreactivity which then is visible in several fractions. Still, all of these metabolites are OT related and thus can be referred to as immunoreactive oxytocin metabolites (iOTM).

By comparing the immunoreactivity of the OT standard to that found in dog and wolf urine, we found that most of the immunoreactivity in the dog and wolf urine matches what was found for the extracted iuOTM when using the Enzo Assay. However, for both species, the assay captured immunoreactivity in fraction 8. This peak did not coincide with immunoreactivity found for the OT standard. While one study reported one single immunoreactive peak (congruent with synthetic OT) in extracted human urine (Amico et al., 1987), other studies found multiple immunoreactive peaks, for example in extracted human serum (Szeto et al., 2011) and extracted prairie vole plasma (Carter et al., 2007), both using RIA and EIA. The vole study found one of the peaks not found for the OT standard to be congruent with arginine-vasopressin (when measured with RIA). But both studies were unable to identify most, if not all of the non-OT peaks. The study on human serum even reported that the fraction that co-eluted with authentic OT (standard OT) did not correspond with the major peak of immunoreactivity, raising concern about the immunospecificity of the antibody of the assay and suggests that these could be oxytocinase degradation products (Szeto et al., 2011). Furthermore, all three studies reported one single peak for OT standard, at least two of these studies used commercially available OT standard for fractionation, but none reported whether the OT standard was extracted before fractions were collected (Carter et al., 2007; Szeto et al., 2011). These differences in methodologies may contribute to the discrepancies in findings between these studies and the present study; namely that here we used extracted standard delivered with the assay system. To further investigate these discrepancies, we collected and assayed fractions of an unextracted OT standard sample. Comparing the immunogram of the extracted to the unextracted standard revealed immunoreactivity to be present in several fractions for both, but with two peaks in the extracted and one peak in the not extracted OT standard (see Fig. SI 6). These results suggest that the extraction method altered OT structure leading to a shift in the retention times for immunoreactive substances potentially by the formation of dimers caused by the elevated temperatures during extraction (e.g., Hawe et al., 2009). Interestingly, this discrepancy between immunoreactivity peaks in unextracted standard and radioactivity peaks in earlier HPLC fractions was already found for marmoset urine (Seltzer and Ziegler, 2007), indicating that despite this shift in immunoreactivity to earlier fractions, the assay antibody mainly cross reacts with substances stemming out of the OT metabolism. Overall, our results suggest acceptable immunospecificity of the Enzo Assay for detecting iuOTM in dogs and wolves.

Ideally, a radiometabolism study should be performed to identify metabolites that stem from the oxytocin metabolism. Radiometabolism studies provide detailed information on excretion routes and patterns of hormones. To achieve this, the radioactively labeled hormone of interest is injected into the animal and relevant samples of matrices, in which the hormone is to be assessed (e.g., blood, urine, feces), are collected repeatedly. This allows the assessment of the time lag between administration and excretion, and an estimation of main excretion pathways. Analyzing these samples with HPLC, with the purpose of assessing radioactivity within fractions of one sample, permits the identification of the specific OT metabolites or other, not OT derived compounds that are measured by OT-EIAs. In a study on common marmosets radioactive OT was injected into 4 adult male individuals and subsequently, urine was collected for 48 h following injection (Seltzer and Ziegler, 2007). Radioactivity was present in fractions of extracted urine samples congruent with an unextracted OT standard (following HPLC separation of samples), but also, and to a higher degree, that radioactivity was found in fractions where no unextracted OT standard was found. The authors conclude that OT can be measured in marmoset urine samples, but also that OT might undergo major metabolic breakdown or deposit in other tissues of the body (as suggested by a study on rats Aroskar et al., 1964). However, as metabolic systems differ across animal species such tests need to be conducted for each species separately.

However, such studies are time consuming and require an animal shelter and a laboratory equipped for working with radioactivity. Moreover, the administration of the radioactively labeled hormone requires invasive procedures and the feasibility of these procedures will depend on the species and its protection status.

4.2. Repeatability

If results of hormonal measurements are not reproducible, a biologically meaningful interpretation can be difficult or impossible, potentially leading to wrong conclusions. Reproducibility has been assessed for various steroid hormones in plasma, however, as peptide hormones are more susceptible to degradation in relation to e.g., storing conditions, reproducibility might comparably be poorer.

Here we found that measuring the same sample repeatedly out of different aliquots led to a higher variance across iuOTM measurements compared to when derived out of the same aliquot. However, the variance of 15.36% for the repeated measurements out of different aliquots is still in an acceptable range. Our results indicate that storage can affect individual samples and even aliquots of the same sample differently, therefore, the impact of storage conditions on hormone levels need to be routinely investigated when working with a new species or matrix and if necessary storage methods need to be further improved.

4.3. Storage & stability

4.3.1. Short-term storage of dog urine samples at 4 °C or room temperature

As hormones, due to e.g., the presence of bacteria in biological specimen, are prone to degradation, concentrations of hormones or their metabolites might change with treatment and storage duration (Buchanan and Goldsmith, 2004). Thus, the treatment and storage of samples following collection is a major issue. Here we examined the effect of short and long term storage on iuOTM levels as well as the effect of adding a preservative to the urine sample following collection. Our results indicate that iuOTM levels decrease, the longer dog urine samples are stored at 4 °C or at room temperature before being frozen for long term storage. More specifically, changes in iuOTM levels are within normal assay variability when stored no longer than 6 h at 4 °C and no longer than 1 h at room temperature. Additionally, adding phosphoric acid, to prevent OT degradation, immediately following sample collection, led to slightly less variance across time points. This indicates that the addition of phosphoric acid to urine samples prior to storage may improve the comparability of iuOTM levels across samples. Fluctuating concentrations depending on storage condition have been reported previously for other biomarkers (Behringer et al., 2017; Heistermann, 2010; Toone et al., 2013). Thus, as has been suggested for other hormones, especially for peptide hormones (Heistermann, 2010), we recommend freezing urine samples immediately following collection. In addition, even though variance in iuOTM levels were within normal variability of the assay system (as indicated by the assay provider), adding phosphoric acid as a preservative may help to avoid an increase in fluctuating iuOTM levels. However, if needed, acidified urine samples can briefly be stored at 4 °C until they can be frozen and stored for further analysis.

4.3.2. Exposure of dog and wolf pooled urine samples to repeated thawing

Repeatedly thawing samples, which can happen accidentally during transport of samples from e.g., field stations to laboratories, where samples get analyzed or intentionally, when samples need to be remeasured during analysis, have been shown to affect levels of biomarkers (Behringer et al., 2017; Reyna et al., 2001). While such degradation effects were also found for OT measured in human urine (Reyes et al., 2014) OT levels were unaffected by repeated freeze-thaw cycles in human plasma (Szeto et al., 2011). We did not find an obvious effect of repeated freeze-thaw cycles on iuOTM levels of dogs and wolves. This indicates that iOTM can be measured in urine samples of dogs and wolves even when samples were exposed to repeated thawing as long as exposure to room temperature is limited to short time periods.

4.3.3. Long term storage of dog and wolf urine

Studies that re-assessed levels of biomarkers after months or years of storage have reported varying levels of the respective biomarker compared to initial measurements (e.g., Heistermann and Higham, 2015; Kesner et al., 1995; Khan et al., 2002; Schaebs et al., 2016). In addition, a previously conducted study found that the recovery of human urinary OT decreased by 10% within two weeks of storage at −20 °C (Amico et al., 1987) and thus extracted and assayed all samples at the day of collection. This is of course not feasible for most studies, especially when conducted on wild or semi-wild living animals. Our results show that urinary OT levels increase with increasing storage duration, independent of storage condition. However, variability across samples was smallest when samples were stored as ethanol extracts at −20 °C. Based on these results, it seems recommendable to store urine samples at −80 °C until extraction, extract those samples as soon as possible, then store them in ethanol at −20 °C until further processing in the lab and aim at measuring all extracts belonging to the same study after a comparable storage duration. In case this is impossible, storage duration could be included as a control variable in the statistical analysis or discrepancies in storage duration should at least be reported to allow the reader to estimate a potential impact of storage duration on the results.

4.4. Effect of concentrating standard on iuOTM levels

Concentrating samples during extraction often becomes of interest when the target hormone is only present in low concentrations. One previous study already found that human urinary OT levels might vary, when measured at different concentrations (Amico et al., 1987). We found that urinary OT levels were underestimated when samples were concentrated. This effect could be either due to matrix effects of unknown substances influencing antibody binding patterns on the assay or due to concentration dependent sorbent holding capacities of the SPE cartridges when extracting the samples. However, we found that OT levels of standard in assay buffer were as well underestimated when concentrated. This indicates that the effect was not only caused by potential matrix effects but to a significant degree also by concentration dependent differences in sorbent holding capacities of SPE cartridges. Since for a number of species, urinary OT levels are relatively low, necessitating a certain level of concentration, a solution could be to at least measure all samples collected within the same research project using the same concentration factor. Again, this pattern needs to be further investigated and extraction methods need to be improved.

4.5. Conclusion & general considerations

In summary, the results of this study suggest that the assessment of iuOTM in dogs and wolves with the tested extraction method and EIA is possible, if a number of precautions are respected. While parallelism revealed acceptable results, recovery tests resulted in a considerably high level of variance, especially in the lower range of the assay. Graphed immunograms suggest acceptable immunospecificity of the Enzyme Immunoassay, however, also revealed the presence of other substances causing immunoreactivity in the urine of dogs and wolves. Together, the immunograms indicate that OT is present in urine samples of dogs and wolves, but also that OT might undergo metabolic changes or breakdown and that those products of OT (or OT like substances) might be caught by the antibody of the assay system with less affinity (see also Ziegler (2018)). Furthermore, our results show variable reproducibility of iuOTM measurements, when the same samples are extracted and measured repeatedly indicating substantial room for further improvement of storage methods. However, special care needs to be taken with regard to short and long term storage. Urine samples should be stored at 4 °C until they can be aliquoted and frozen at −80 °C. For long term storage, it is recommendable to extract samples and store extracts in 100% ethanol as soon as possible following collection at −20 °C until further analysis. Finally, as shown by our results, OT levels appear to be underestimated when a concentration step is added to the extraction procedure. Therefore, to retain comparability we recommend that all samples within a study should be measured at the same concentration. Even though there is growing criticism regarding the validity of OT measurements (e.g., Horvat-Gordon et al., 2005; Leng and Sabatier, 2016; McCullough et al., 2013; Szeto et al., 2011), there is still a lack of proper method validation. Validating a method is of crucial importance to guarantee that the assay system at hand reliably and repeatedly measures the biomarker of interest (Buchanan and Goldsmith, 2004; Heistermann, 2010), especially if potentially elaborate and expensive studies, including a large number of individuals, are conducted. However, commercially available Enzyme Immunoassays are very expensive, which unfortunately may at least partially contribute to the lack of validation studies. It is therefore of paramount importance when planning a study to include an adequate proportion of the resources to method validation and reviewers of grants applications should estimate if indeed requested resources are covering this important part of a project. Particularly problematic are studies where the principal investigators have no background in endocrinological laboratory methods but rather send their samples to a service lab and just receive the results without any specifications. These labs rarely have the resources to carry out time consuming validation experiments and therefore serious methodological problems might go unnoticed. Here journal editors need to choose reviewers carefully to not only cover the expertise of the ultimate interpretation of the results but as well the soundness of the laboratory standards. Another source of concern is the lack of sufficient reports of at least basic quality measures, such as extraction efficiency and assay accuracy or how samples that fell outside of the linear range of the assay or non-matching duplicates were handled in the laboratory and during analysis. If this information is not provided, it prevents the reader from being able to critically evaluate the relevance of the published results. Even if, as in other cases, quality parameters might not be optimal, reporting them will allow the reader to set quality parameters in relation to effect sizes and draw conclusions about the explanatory power of the presented results.

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Appendix A. Supplementary data

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