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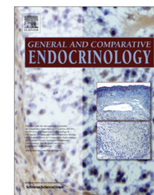

There is no correlation between glucocorticoid receptor mRNA expression and protein binding in the brains of house sparrows (*Passer domesticus*)

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ABSTRACT

The stress response represents an animal's attempt to cope with a noxious stimulus through a rapid release of corticosterone or cortisol (CORT) into the bloodstream, resulting in a suite of physiological and behavioral changes. These changes are mediated in large part through CORT's binding to two different intracellular receptors, the high-affinity mineralocorticoid receptor (MR) and the lower-affinity glucocorticoid receptor (GR). We tested the hypothesis that GR and MR mRNA expression would correlate with functional protein expression in neuronal tissue of wild-caught house sparrows (*Passer domesticus*). To test this hypothesis, we performed a parallel procedure in which protein concentrations were quantified in one half of house sparrow brains ($n = 16$) using radioligand binding assays, and mRNA levels were quantified in the other brain half using reverse-transcriptase quantitative PCR (RT-qPCR). Two reference genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and TATA-box binding protein (TBP), were used for relative quantification of GR and MR mRNA. Quantifications showed that these two reference genes were not correlated with each other. Furthermore, there was no correlation between mRNA and protein levels for GR or MR using either reference gene, suggesting that regulation of mRNA and protein levels for MR and GR is not tightly linked. This study provides insight into the importance of regulatory steps between mRNA expression and the creation and stability of a functional protein. The overall conclusion is that mRNA expression cannot be used as a proxy for GR or MR binding in house sparrows.
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1. Introduction

The physiological stress response represents an organism's attempt to maintain homeostatic balance when faced with a noxious stimulus, or stressor, in its environment (Sapolsky et al., 2000). Unlike the "fight-or-flight" response, which occurs almost instantaneously, physiologically-relevant increases in corticosterone (CORT) occur more slowly, within 2–3 min after exposure to a stressor (Romero and Reed, 2005). This CORT increase helps the organism cope with stressors and primes its defenses should another stressor arise (Sapolsky et al., 2000).

The stress response involves a multitude of effects, including increases in circulating blood glucose and activity levels, as well as the inhibition of systems not essential for immediate survival such as growth, reproduction and immune function (Romero, 2004; Sapolsky et al., 2000). Many of these physiological effects are the result of transcriptional activation of glucocorticoid receptors (Joëls et al., 2008; John et al., 2009; Zennaro et al., 2009). In birds and mammals, there are two intracellular corticosteroid receptors that bind to CORT, as well as a possible membrane-bound receptor that is not the focus of this study (Breuner and Orchinik, 2009; Breuner et al., 2003; Funder, 1997; Orchinik et al., 1991; Schmidt et al., 2010). The mineralocorticoid receptor (MR) has an approximately 10-fold higher affinity for CORT than the glucocorticoid receptor (GR) (Breuner and Orchinik, 2009; de Kloet et al., 1998). Both are transcription factors, meaning they bind to specific glucocorticoid response elements in DNA, where they promote transcription of certain genes and repress transcription of others (Funder, 1997; John et al., 2009). For example, in laboratory rats, activated GRs and MRs altered expression of 70–100 genes in the hippocampus alone (de Kloet et al., 2005).

The difference in affinities of the two CORT receptors may allow for their differential regulation by changes in circulating hormone concentrations (McEwen et al., 1986). With a low dissociation constant (K_d) of ~0.2 nM, MR is thought to be nearly constitutively activated by circulating concentrations of baseline CORT (Landys et al., 2006; Reul et al., 1987). In mammals, the role of MR in the brain includes maintenance of stress-related neuronal circuit integrity, appraisal and organization of sensory information, and general permissive actions of the stress response (de Kloet et al., 2005; Joëls et al., 2008; Sapolsky et al., 2000). Elsewhere in the

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body, MR activated by CORT mediates effects related to metabolism and the immune system (Landys et al., 2006; McEwen et al., 1997; Zennaro et al., 2009). With a higher K_d of ~6 nM, GR is thought to be primarily occupied by high CORT levels found during the circadian maximum or in response to a stressor (Landys et al., 2006; Reul et al., 1987). GR has many different roles in the body related to energy regulation, immune function and reproduction (Landys et al., 2006; McEwen et al., 1997; Rivier and Rivest, 1991); in the mammalian brain, it also functions in consolidating and storing information relevant to stressful events for future use in similar situations (de Kloet et al., 2005).

While it is the MR and GR proteins that bind CORT and cause the effects of the physiological stress response, many researchers have also chosen to look at upstream targets, including mRNA expression. Recent advances in the reverse transcription quantitative polymerase chain reaction (RT-qPCR) have reduced the time and cost of this technique, making mRNA quantification easier than ever (Ponchel et al., 2003). Because of this, fields such as transcriptomics, the study of all the RNA products in an organism, are gaining attention as one way to better understand the stress response.

In a recent review, however, Feder and Walser, (2005) caution that one major drawback of transcriptomics is that knowledge of mRNA expression does not necessarily equate to knowledge of functional protein expression in that organism. Indeed, in species ranging from humans to bacteria, mRNA and protein levels have been found to be poorly correlated (Anderson and Seilhamer, 1997; Gygi et al., 1999; Lee et al., 2003; Washburn et al., 2003). This may be because there are a number of regulatory processes downstream of mRNA synthesis, including mRNA degradation and sequestration, which can prevent the transcript from being made into functional protein; some of these regulatory steps are controlled by small RNA pathways (Valencia-Sanchez et al., 2006; Watson, 2008). There are also downstream regulatory steps, such as RNA stabilization, that can increase the amount of protein made from a single transcript, which could also create mismatches between mRNA and protein levels. However, while the general consensus is that protein and transcript are not always correlated (Greenbaum et al., 2003), they can be in certain situations (e.g. White et al., 2004).

Researchers have tested a number of different genes in a variety of species to determine whether mRNA and protein levels are correlated. The emerging pattern appears to be dependent on both the organism and the gene in question. In the handful of studies examining this question for CORT receptors, GR protein levels do not seem to show particularly strong correlations with mRNA levels. For example, in rainbow trout (*Oncorhynchus mykiss*), Vijayan et al. (2003) demonstrated that CORT implants resulted in significantly higher GR mRNA abundance, despite a drop in GR protein content. In the hippocampus of laboratory rats exposed to stressors, Herman et al. (1999) discovered that GR protein increased despite mRNA levels remaining unchanged. However, this question has been examined by only a few studies in a small number of species, and primarily with GR rather than both CORT receptors. Given the growing interest in understanding the stress response in free-living animals, and the importance of receptors in mediating this response, it would be useful to determine whether transcript and protein for both GR and MR were correlated in a wider variety of wild species. If so, then mRNA expression could be used as a proxy for protein expression, avoiding some of the challenges associated with quantifying protein (Greenbaum et al., 2003).

In this study, we used a parallel protocol that allowed for the determination of total mRNA and protein binding of both GR and MR in the two brain halves of the house sparrow (*Passer domesticus*). To our knowledge, this is the first time anyone has tested for correlations between protein and transcript for CORT receptors in any wild avian species, although other studies have examined patterns in GR and MR mRNA in free-living birds with treatments such as chronic stress (Dickens et al., 2011) and life history stage (Liebl et al., 2012). In one half of the brain, we used radioligand binding assays to quantify protein expression, and in the second half of the same brain we used RT-qPCR to quantify mRNA expression. Furthermore, because the choice of reference gene can have large impacts on quantitative PCR results (Bas et al., 2004; Huggett et al., 2005; Radonic et al., 2004), we performed two sets of analyses that calculated relative mRNA expression using two different reference genes.

2. Methods

2.1. House sparrow capture and perfusion

Wild house sparrows were caught 3–8 December 2010 ($n = 8$), 31 March to 7 April ($n = 8$) and 23–24 May 2011 ($n = 8$) in Medford, Massachusetts using Potter traps and mist nets at bird feeders. We chose house sparrows as the study species because receptor-binding techniques have been validated for use in this species (Breuner and Orchinik, 2001) and intracellular CORT receptors have already been fully characterized in house sparrow brain (Breuner and Orchinik, 2009). Sparrows were housed in the laboratory in pairs and given mixed bird seed, grit and water *ad libitum*. On days 5 and 6, they were chemically adrenalectomized using two injections of mitotane (ortho, para, dichlorodiphenyl dichloroethane, Sigma–Aldrich, St. Louis, MO). In house sparrows, mitotane is effective in blocking endogenous CORT production that could otherwise interfere with radioligand binding assays (Breuner et al., 2000; McEwen et al., 1974). Birds received two 100 μl intramuscular injections of mitotane (180 mg/kg body weight) dissolved in peanut oil using sonication (Branson Sonifier 450, Danbury, CT), and were given chopped apples to compensate for any potential inability to mobilize glucose.

On the morning of day 7, birds were placed in cloth bags for 30 min and ~50 μl of blood taken to ensure that mitotane treatment was successful. All blood samples were stored on ice until centrifugation, and then plasma was removed and stored at −20 °C. We determined CORT concentrations in each sample using radioimmunoassay (RIA) following the methods of Wingfield et al. (1992). In all birds used for this study, mitotane treatment brought stress-induced CORT titers down to less than 5 ng/mL plasma (compared to ~20–30 ng/mL for house sparrows not treated with mitotane (Romero et al., 2006). Anesthetized birds were transcardially-perfused using ice-cold heparinized saline to remove circulating corticosterone binding globulin, which could otherwise interfere with MR and GR binding in the radioligand binding assays. Whole brain samples were cut in half along the medial longitudinal fissure and flash-frozen on dry ice immediately upon removal and stored at −80 °C for long-term storage. All procedures were performed according to AALAC guidelines and approved by the Tufts University Institutional Animal Care and Use Committee.

2.2. Validation experiment

An important assumption of our experimental protocol is that protein binding and mRNA expression will be equivalent in both brain hemispheres. To test this assumption, we performed a validation experiment with the first 8 sparrows caught where we ran both brain hemispheres either through the receptor binding assay ($n = 4$; see protocol below) or the RT-qPCR procedure ($n = 4$; see below) and compared values between the two sides of the same brain.

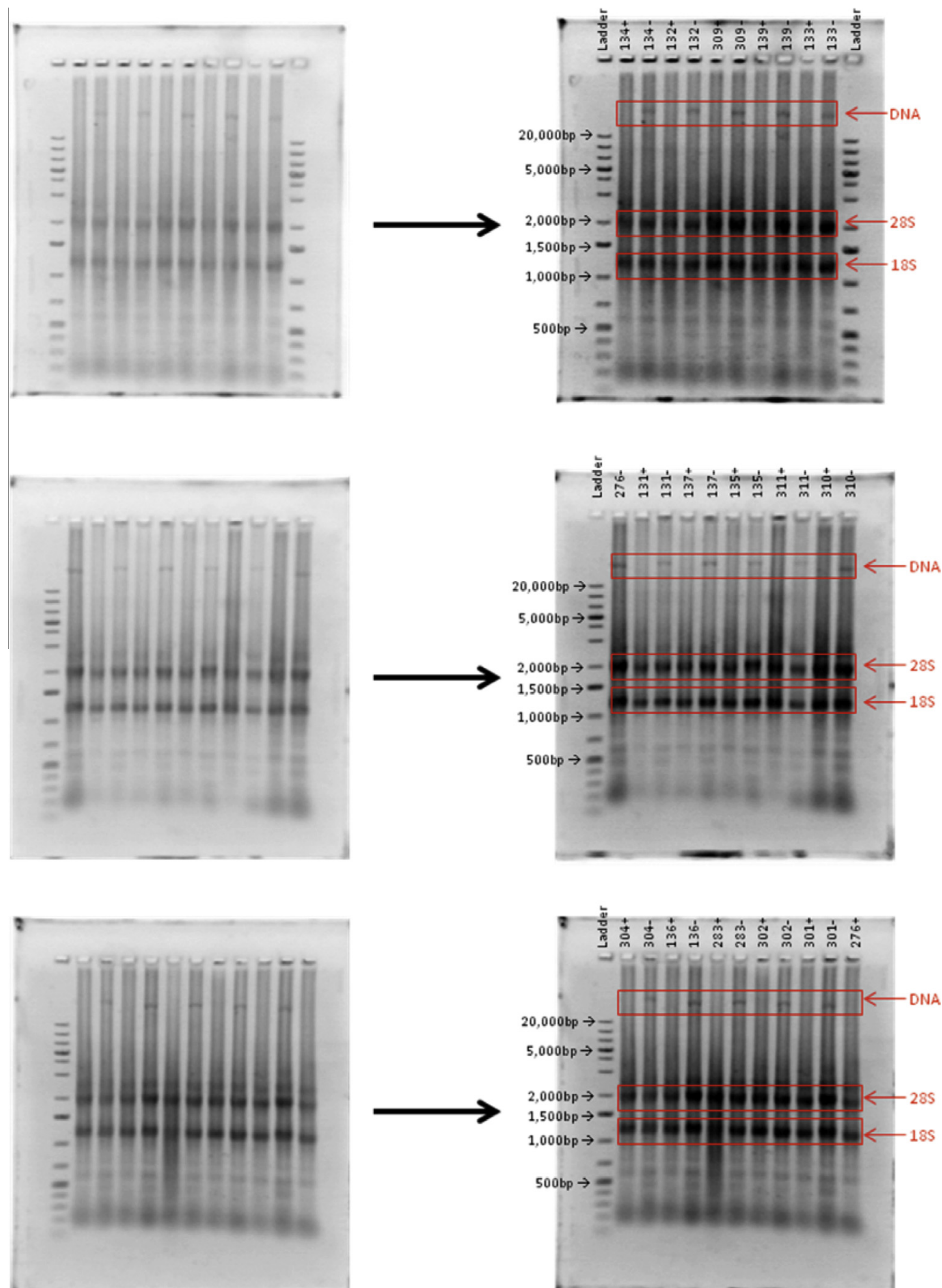

Fig. 1. Agarose-gel electrophoresis of RNA extracted from house sparrows ($n = 16$), checking for RNA purity. Digital image contrasts were adjusted on Adobe Photoshop CS5 to make bands more distinct (right panel). Clear 18S and 28S rRNA bands can be seen on all gels at 1250 and 2000 bp, respectively. This is lower than their known size of 1900 and 5000 bp for the 18S and 28S subunits, respectively, possibly due to some RNA degradation. Large DNA bands greater than 20,000 bp can be seen in all −DNase controls and are not present in any +DNase samples, showing successful removal of DNA contamination.

2.3. Cytosol preparation

One half of each brain was randomly selected for use in the radioligand binding assay to determine receptor binding for total GR and MR. Cytosol was prepared following the methods of Breuner and Orchinik, (2001). Briefly, brain tissue was homogenized in a glass-Teflon homogenizer (VWR, Radnor, PA) with 0.5 mL TEGMD buffer/0.11 g tissue (10 mM Tris base, 1 mM EDTA, 20 nM sodium molybdate dihydrate, 10% glycerol, and 5 mM dithiothreitol, Sigma Aldrich, St. Louis, MO). The homogenized tissue was then mixed with an equal volume of Dextran-coated charcoal in TEGM (1% Norit-A charcoal, Sigma Aldrich; 0.1% Dextran, Pharmacia Biotech AB, Uppsala, Sweden). The samples were spun at 105,000 g for 1 h at 4 °C in an ultracentrifuge and the cytosolic supernatant collected for immediate use in the radioligand binding assay.

2.4. Radioligand binding assay for receptor quantification

Specific binding of receptors (in fmol/mg protein) was determined using a radioligand binding assay adapted from Breuner and Orchinik, (2001) and described in detail elsewhere (Lattin et al., 2012). Briefly, cytosol was incubated with 10 nM ^{3}H-CORT (Perkin Elmer, Waltham, MA) and either TEGM buffer, 1 μM unlabeled CORT (Sigma Aldrich), or 1 μM RU486 (mifepristone, Tocris

Bioscience, Minneapolis, MN) for 4 h at 22 °C. The TEGM buffer estimates total ^{3}H-CORT binding, the unlabeled CORT competitor estimates non-specific binding, and the RU486, which binds with high affinity to GR only, estimates MR binding. Based on affinity estimates derived from previous equilibrium saturation analyses (Lattin et al., 2012), the law of mass action predicts that 10 nM ^{3}H-CORT should occupy >95% of MR and ~63% of GR.

After incubation, samples were filtered on a Brandel harvester (model M24, Gaithersburg, MD), and rinsed with 3 × 3 mL ice-cold TEM buffer (5 mM Trizma base, 1 mM EDTA, 10 mM sodium molybdate dihydrate, pH 7.45 at 4 °C). Filters were placed into individual scintillation vials and 5 mL scintillation fluid (Ultima Gold, Perkin Elmer) added to each vial. The vials were vortexed and allowed to sit at least 12 h before counting on a Packard 1600 TR Liquid Scintillation Analyzer. Binding in individual samples was standardized per mg protein using a Bradford assay with bovine serum albumin as a standard (Sigma Aldrich).

2.5. RNA extraction

RNA was extracted from each brain half using TRIzol® Reagent (Ambion, Life Technologies, Carlsbad, CA) according to the manufacturer's procedure. Briefly, tissue samples were weighed and homogenized in 1 mL TRIzol/100 mg tissue on ice, then incubated at room temperature for 5 min. The TRIzol-tissue mixture was mixed with chloroform (Sigma–Aldrich), incubated at room temperature for 3 min, and centrifuged to separate the aqueous layer. The aqueous layer was removed and mixed with isopropyl alcohol (Thermo Fisher Scientific, Waltham, MA), incubated for 10 min at room temperature, and centrifuged to form an RNA pellet. The isopropyl alcohol was removed and the pellet was washed with 75% ethanol (Thermo Fisher Scientific). The pellet was allowed to air-dry for up to 20 min to evaporate remaining ethanol, and re-dissolved in DEPC-treated water (Thermo Fisher Scientific). RNA from each individual was separated into aliquots and stored at −80° C for up to 1 month before further processing.

2.6. DNase treatment

A DNase treatment was applied to RNA samples to remove any contaminating DNA, using the TURBO DNA-free kit (Ambion) according to the procedure described in the manual. Briefly, kit buffer and TURBO DNase were added to all but one RNA aliquot per individual. The last RNA aliquot of each house sparrow was treated with buffer and DEPC-treated water to act as a −DNase control for evidence of effective DNase treatment.

RNA concentrations and purity were measured using a NanoDrop 2000 (Thermo Fisher Scientific). All RNA used for reverse transcription had an optical density 260/280 ratio greater than 1.9, lower than that of pure RNA (which is ~2.0) but higher than the ratio of 1.8 generally used as an indicator of acceptable RNA quality (Fleige and Pfaffl, 2006). It has also been shown that for RT-qPCR products 70–250 bp long (ours fall within this range), results are considered relatively independent of RNA quality (Fleige and Pfaffl, 2006).

2.7. Gel electrophoresis of RNA samples

One aliquot of DNase-treated RNA per house sparrow was run alongside its −DNase control on a 1% UltraPure Agarose gel (Invitrogen, Life Technologies, Carlsbad, CA) in TBE buffer (45 mM Tris-borate, 1 mM EDTA, Sigma Aldrich). Sample, DEPC-treated water and Orange DNA Loading Dye (Fermentas, Thermo Fisher Scientific, Waltham, MA) were loaded into each well and run alongside an appropriate ladder for comparison (O'Gene Ruler, 1 kb Plus DNA Ladder, ready-to-use, Fermentas). Gels were run for 1.5 h at 80 V (EC 105, E-C Apparatus Corporation, Milford, MA) and stained with Instastain ethidium bromide sheets (Edvotek, Bethesda, MD). Ethidium bromide-stained bands were visualized and photographed using the Foto/UV 26 system with Foto Analyst/PC Image software (version 10.21, Fotodyne Incorporated, Hartland, WI). Gel electrophoresis showed that the removal of endogenous DNA was successful (Fig. 1); while DNA bands are clearly visible on −DNase samples, no such bands appear on +DNase samples.

2.8. Reverse transcription of RNA to cDNA

Reverse transcription of RNA samples was performed using a High Capacity RNA-to-cDNA kit (Applied Biosystems, Life Technologies, Carlsbad, CA) according to the procedure described in the manual. Briefly, DNase-treated RNA was added to a reaction containing kit buffer, reverse transcriptase (RT) enzyme mix (excluded in −RT controls), and DEPC-treated water. All RNA samples were diluted to the same concentration using DEPC-treated water (quantified using a NanoDrop 2000). The reaction was run at 37 °C for 60 min, 95 °C for 5 min, and held at 4 °C for up to 1 h. cDNA products were stored at −20 °C and thawed a maximum of two times for use in qPCR.

2.9. Primer design

Custom primers for house sparrow GR, MR, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and TATA-Box Binding Protein (TBP) genes were designed using Primer3 software developed at the Massachusetts Institute of Technology (Rozen and Skaletsky, 2000) using partial coding sequences provided by the NCBI GenBank (http://www.ncbi.nlm.nih.gov, Table 1). Based on the recommendations of several authors (Bustin, 2002; Huggett et al., 2005; Vandesompele et al., 2004), we chose to use multiple reference genes. We chose GAPDH as our first reference gene due to its frequent use in many different studies (Barber et al., 2005), although there is evidence that its expression may vary depending on experimental treatment (Bas et al., 2004). We chose TBP as a second reference gene, as it has been previously used as a reference gene in house sparrow brain (Helfer et al., 2006), and there is evidence its expression may be more stable (Radonic et al., 2004). Multiple unique primer sets were designed for each gene using recommendations from Bustin, (2000). Two primer sets per gene were chosen

Table 1
Primers used for reverse-transcriptase quantitative PCR in house sparrow brain.

Name	Sequence (5′–3′)	GenBank accession number	Primer efficiency (%)
GR1-Fwd	catccacaacctcagcaacta	GU229783	93.6
GR1-Rev	aagtgttcttcaagagggcc		
GR4-Fwd	cctggaatgaggtcagatgt		99.9
GR4-Rev	caactacgggaccacctc		
MR1-Fwd	cagctcagcttcgaggagta	GU229784	98.2
MR1-Rev	acagttcccaaggatggtct		
MR4-Fwd	tgtcatcgtttgccttgagt		90.5
MR4-Rev	ttttgctccagacctgatctt		
GAPDH1-Fwd	cccctaacgtgtctgttgtg	AF416452	99.5
GAPDH1-Rev	tagtgaaggctgctgctgat		
GAPDH4-Fwd	tcatacacagaggaccaggtt		93.8
GAPDH4-Rev	tttgtcaagctggtttcctg		
Tbp1-Fwd	tcctctccaatgactccaat	AY158633	94.0
Tbp1-Rev	taggtcaagtttgcaaccaa		
Tbp4-Fwd	cagggaacatctggtcaaac		93.2
Tbp4-Rev	tggagtcattggagtcattg		

to perform qPCR based on melting curve data, primer efficiencies, and specificity of amplification confirmed with gel electrophoresis and sequencing of qPCR products (see below). Primers were purchased from Invitrogen, and stored as concentrated stock solutions in TE buffer (10 mM Tris Cl, 1 mM EDTA, Sigma Aldrich) at −20 °C.

2.10. Determining primer efficiencies

Primer efficiencies were calculated for each primer set (Hoebeeck et al., 2007). A cDNA sample of known concentration (determined using a NanoDrop 2000) was serially diluted 1:5 in DEPC-treated water and run using qPCR for each primer set. The resulting C_t was plotted against the log concentration of cDNA. The slope of this line, when used with the equation $(10^{(-1/-\text{slope})}) - 1$, gave the primer set efficiency. A slope of −3.32 would indicate an efficiency of 100%. All primer sets used had efficiencies greater than 90%. Dissociation curves were also generated in order to check the specificity of the primers and check for primer dimers. All primer sets used showed single sharp peaks in dissociation curves (data not shown).

2.11. qPCR procedure

Relative qPCR was performed using the 7300 Real Time PCR System with SDS software and Power SYBR Green PCR Master Mix (Applied Biosystems). 96-Well Optical Reaction Plates were used and sealed with MicroAmp Optical Adhesive Film (Applied Biosystems). Prior to final qPCR analysis, samples were run at 1:10, 1:25 and 1:50 dilutions in DEPC-treated water to determine optimal cDNA concentrations. For final analyses, the 25 μL reaction volume in each well contained 1.5 μL of 1:50 diluted cDNA, 25 nM each forward and reverse primers, and 12.5 μL of SYBR Green Master Mix. The reaction was run under standard thermocycling conditions: Stage 1: 1 rep, 2 min at 50 °C, Stage 2: 1 rep, 10 min at 95 °C, Stage 3: 40 cycles of 15 s at 95 °C followed by 1 min at 60 °C.

Each plate contained mRNA from a single house sparrow with GR and MR quantified against one of the two reference genes, such that there were 32 plates total (one GAPDH and one TBP plate per house sparrow). Each cDNA sample was run alongside a −RT control (which underwent DNase treatment, but had no enzyme added during the reverse transcriptase step) as well as a blank with DEPC-treated water. The −RT control acted as a no-template control to ensure that all endogenous DNA was removed during DNase treatment. The water control was used to ensure there were no primer dimers or outside DNA contamination. All samples were run in triplicate and the average threshold cycle (C_t) and standard deviation was used for analysis. A manual average threshold was used across all plates per reference gene for normalization of C_t values. Relative quantification was performed using the 7500 SDS software, with one individual house sparrow used as the calibrator (so RQ = 1.0) for all studies.

2.12. Gel electrophoresis and sequencing of qPCR products

Amplified qPCR products were run on a 3% UltraPure Agarose gel in TBE Buffer. Sample, DEPC-treated water and Orange DNA Loading Dye were loaded into each well and run alongside an appropriate ladder for comparison (TrackIt 25 bp DNA ladder, Invitrogen). Gels were run for 90 min at 90 V. Although slightly foggy, the gel showed bands of the expected lengths (Fig. 2, Table 2).

Amplified PCR products were purified with the QIAquick Nucleotide Removal Kit (Qiagen, Hilden, Germany) according to the manual instructions, and sequenced by the Tufts Core Facilities (Boston, MA) with automated ABI 3130XL DNA sequencers (Applied Biosystems). Although we were not able to sequence gene products in their entirety due to small product size, product lost by purification method, and limitations of the sequencers, products of all primer sets used had at least 16 consecutive matching bases to the target mRNA sequence (Table 3).

Table 2
Amplicon lengths from reverse-transcriptase quantitative PCR products as determined by gel electrophoresis. N/A indicates that the primer products were run on a separate gel not shown in Fig. 2.

Primer	Expected amplicon length	Observed amplicon length (approximate)
GR 1	125	130
GR 4	64	70
MR 1	71	75
MR 4	79	85
GAPDH 1	92	100
GAPDH 4	116	125
TBP 1	123	N/A
TBP 4	111	N/A

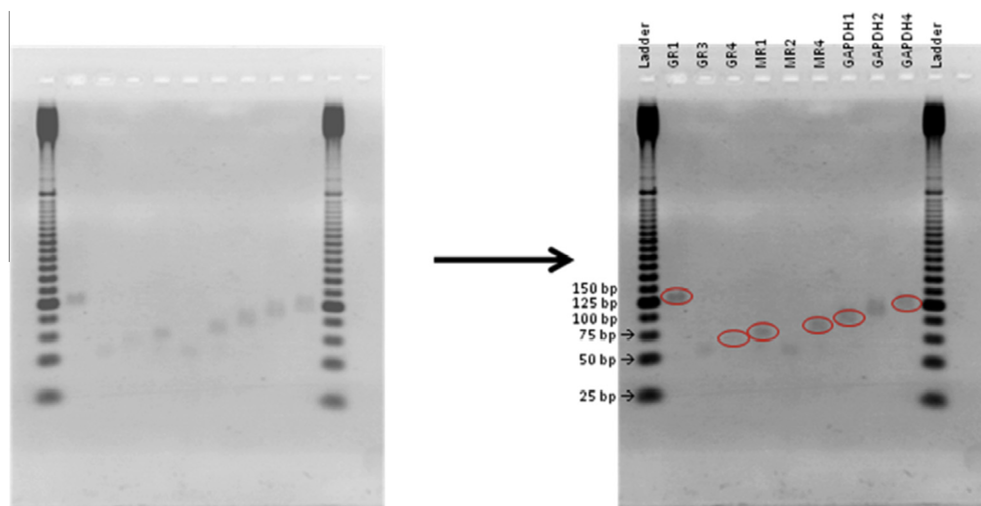

Fig. 2. Agarose-gel electrophoresis of RT-qPCR products. Digital image contrasts were adjusted on Adobe Photoshop CS5 to make bands more distinct (right panel). Gel product sizes were near expected amplicon lengths for all primers (see Table 2). Circled primers indicate those actually used for final qPCR analysis.

Table 3
Sequence data from products of reverse-transcriptase quantitative PCR in house sparrow brain. Amplified PCR products were purified with the QIAquick Nucleotide Removal Kit (Qiagen, Hilden, Germany) and sequenced with automated ABI 3130XL DNA sequencers (Applied Biosystems). We were not able to sequence gene products in their entirety due to small product size, product lost by purification method, and limitations of the sequencers.

Primer set	Sequence homology	Number of bases matching target product
GR 1	GTGCTCCGACGAGGCCTCCGGGTGCCACTACGGCGTCCTGACGTGTGGCAGCTGCAAAGTGTTCTTCAAGAGGG	75
GR 4	ACTACGGGACCACCTC	16
MR 1	CTGCTGCTCTTAAGCACAGTTCCCAAGGATGG	32
MR 4	GCCAGTTCCTCTATTTTGCTCCAG	24
GAPDH 1	AGCCAGCCAGTACGATGACATCAAGAGGGTAGTGAAGGCTGCTGCT	67
GAPDH 4	TTCCTCCACCTTTGATGCGGGCGCTGGCATTGCGCTGAACGACCATTTTGTCAAGCTGGTTT	62
TBP 1	GCTCTGGCATAGTGCCACAACTACAGAATATTGTGTCNACAGTGAATCTTGGTTGCAAACTTGACCTA	68
TBP 4	TCTNACCACAGCCCCTTTACCTGGAACCACACCTCTGTACTCCTCTCCAATGACTCCAATGACTCCAAT	69

2.13. Statistical analyses

All statistical analyses were performed in JMP 8.0 (SAS Institute Inc. 2009). To validate whether a given technique (radioligand binding or RT-qPCR) gave similar values for left and right hemispheres of the same brains, we used two different analyses. First we used a matched pairs analysis to ask the question: are values from the two brain hemispheres using the same technique significantly different? Then we used regression analysis to ask: are values from the two brain hemispheres using the same technique correlated?

To compare GR and MR levels obtained using the two different techniques, we used a rank-order analysis similar to that described by Romero and Reed, (2008). We were interested in determining whether relative protein binding was consistent with relative mRNA expression of GR and MR across all 16 house sparrows. In other words, did a bird with high GR or MR protein binding relative to other birds also have high relative GR or MR mRNA expression? GR and MR were analyzed separately, and all birds were ranked from lowest (16) to highest (1) for both receptor binding and mRNA expression. In each case, we performed a one-way analysis of variance (ANOVA) to look for consistency in ranks between individuals, and also ran regression analyses. We used two primer sets for GR and two for MR, which sometimes gave different rank orders. Thus, we averaged results from the two primer sets, giving a mean mRNA rank per reference gene per receptor. For both GR and MR, we compared ranks of protein binding to mRNA expression using data from both reference genes combined, and also for GAPDH and TBP analyzed separately to determine whether either reference gene showed a stronger correlation. The two reference genes were then compared to each other, to determine whether they gave similar results for a given receptor, e.g., did a bird with high GR using GAPDH as a reference gene also have high GR using TBP?

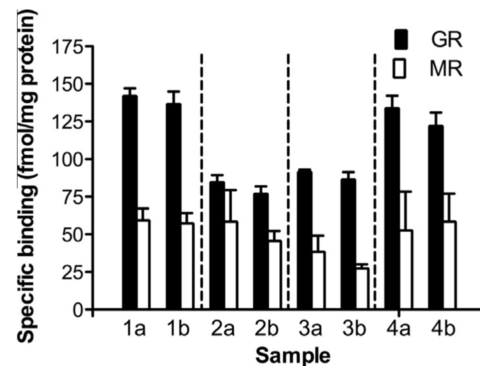

Fig. 3. Radioligand binding data for glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) compared between two hemispheres of the same house sparrow brains ($n = 4$). Samples were run in triplicate, and are labeled by number, where a and b refer to the two different hemispheres of the same brain. All values are presented as means ± SEM.

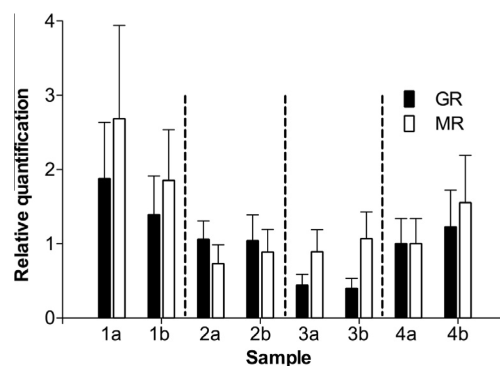

Fig. 4. Relative quantification of mRNA for glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) compared between two hemispheres of the same house sparrow brains ($n = 4$). mRNA was quantified using reverse-transcriptase quantitative PCR with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as a reference gene. Samples were run in duplicate, and are labeled by number, where a and b refer to the two different hemispheres of the same brain. All values are presented as means ± SEM.

3. Results

Using matched pairs analysis, there was no difference between either GR or MR binding ($t = 0.21$, $p = 0.85$, Fig. 3) or mRNA expression using GAPDH as a reference gene ($t = 0.22$, $p = 0.83$, Fig. 4) when we compared the left and right hemispheres of the same brains. Using regression analysis, there were significant correlations between receptor binding ($r^2 = 0.94$, $p < 0.0001$) and mRNA expression ($r^2 = 0.68$, $p = 0.012$) between the left and right hemispheres of the same brains.

There was no correlation between GR protein rank and GR mRNA rank, whether reference genes were compared together or separately (Fig. 5). GR protein rank showed no correlation with mRNA rank when both reference genes were combined ($F_{1,14} = 0.065$, $p = 0.80$, $r^2 = 0.005$), when GAPDH alone was used as a reference gene ($F_{1,14} = 0.004$, $p = 0.953$, $r^2 < 0.001$), or when TBP alone was used as a reference gene ($F_{1,14} = 0.120$, $p = 0.734$, $r^2 = 0.009$).

Similarly, there was no correlation between MR protein rank and MR mRNA rank, whether reference genes were compared together or separately (Fig. 6). MR protein rank showed no correlation with MR mRNA rank when both reference genes were combined ($F_{1,14} = 0.002$, $p = 0.970$, $r^2 < 0.001$), when GAPDH alone was used as a reference gene ($F_{1,14} = 0.087$, $p = 0.772$, $r^2 = 0.006$), or when TBP alone was used as a reference gene ($F_{1,14} = 0.056$, $p = 0.817$, $r^2 = 0.004$).

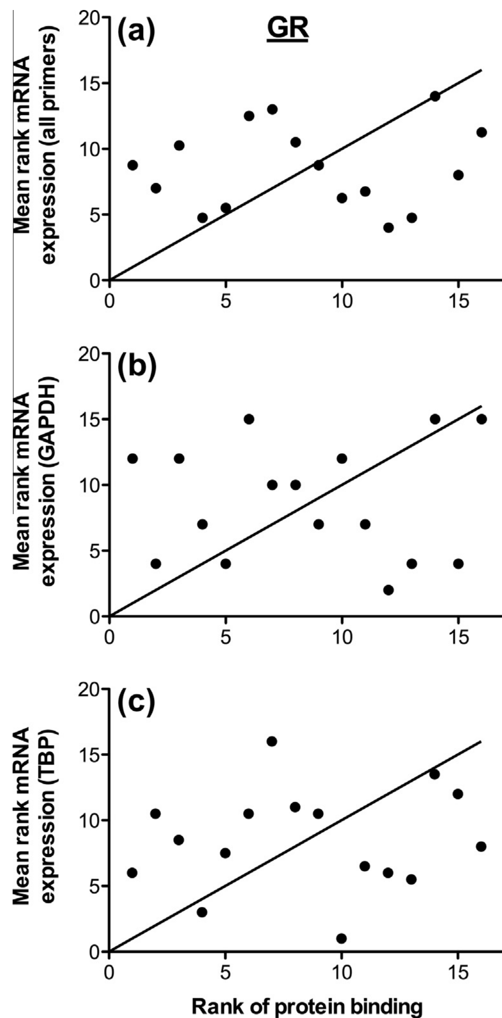

Fig. 5. Rank-order correlations between glucocorticoid receptor (GR) mRNA expression and protein binding in house sparrow brain ($n = 16$). a. Protein binding rank compared to the mean rank given by qPCR using both reference genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and TATA-box binding protein (TBP), with individual comparisons by reference gene found in panels b and c. Individual birds are placed on the *x*-axis in order of lowest to highest GR protein binding rank as determined from radioligand binding assays of one brain hemisphere. The mRNA expression rankings of each bird as determined using RT-qPCR of the other brain hemisphere are placed on the *y*-axis. The ranks using both GR primers compared to a given reference gene were averaged for panels b and c, and the data from both reference genes were averaged for panel a. The solid line indicates a perfect correlation, where the bird with the lowest protein rank of #1 also has the lowest mRNA rank of #1, the bird with protein rank #2 has mRNA rank #2, etc.

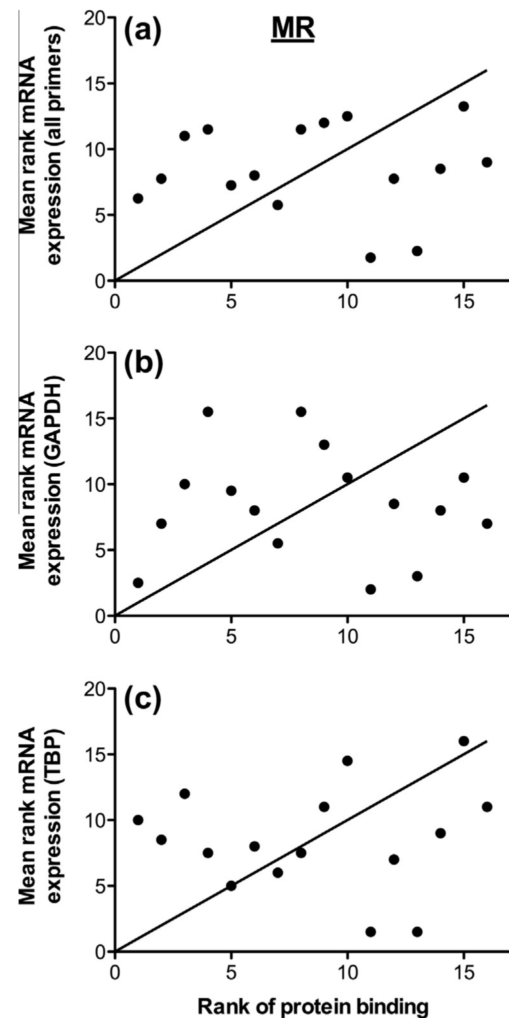

Fig. 6. Rank-order correlations between mineralocorticoid receptor (MR) mRNA expression and protein binding in house sparrow brain ($n = 16$). a. Protein binding rank compared to the mean rank given by qPCR using both reference genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and TATA-box binding protein (TBP), with individual comparisons by reference gene found in panels b and c. Individual birds are placed on the *x*-axis in order of lowest to highest MR protein binding rank as determined from radioligand binding assays of one brain hemisphere. The mRNA expression rankings of each bird as determined using RT-qPCR of the other brain hemisphere are placed on the *y*-axis. The ranks using both MR primers compared to a given reference gene were averaged for panels b and c, and the data from both reference genes were averaged for panel a. The solid line indicates a perfect correlation, where the bird with the lowest protein rank of #1 also has the lowest mRNA rank of #1, the bird with protein rank #2 has mRNA rank #2, etc.

The two reference gene ranks were then compared to each other for GR and MR to determine whether they provided the same information on mRNA expression via qPCR analysis (Fig. 7). There was no correlation between the ranks from GAPDH and TBP reference genes for GR mRNA expression ($F_{1,14} = 0.437$, $p = 0.519$, $r^2 = 0.030$). There was a slight correlation between ranks from GAPDH and TBP reference genes for MR mRNA expression; however, this relationship was not significant ($F_{1,14} = 3.095$, $p = 0.100$, $r^2 = 0.181$).

4. Discussion

We did not find correlations between mRNA expression in one brain hemisphere and protein binding in the other for either GR or MR in wild-caught house sparrows. Because there were two RT-qPCR runs per house sparrow, one using GAPDH as a reference gene and one using TBP, the two sets of mRNA data were also analyzed separately. However, there was still no correlation between mRNA and protein expression ranks for GR or MR when the different reference genes were analyzed separately. The two reference genes were then compared to each other to determine whether they indicated similar mRNA expression levels. Neither the GR nor MR mRNA ranks from the two reference genes were correlated, demonstrating that these two reference genes commonly used with RT-qPCR are not telling the same story. These data suggest that mRNA expression levels do not accurately reflect functional protein levels, and even that different sets of reference genes can yield very different results. The overall conclusion from this study is that mRNA expression cannot be used as a proxy for GR or MR receptor binding in house sparrows.

There were, however, a number of limitations of this study. First was that the house sparrow genome has yet to be sequenced, and

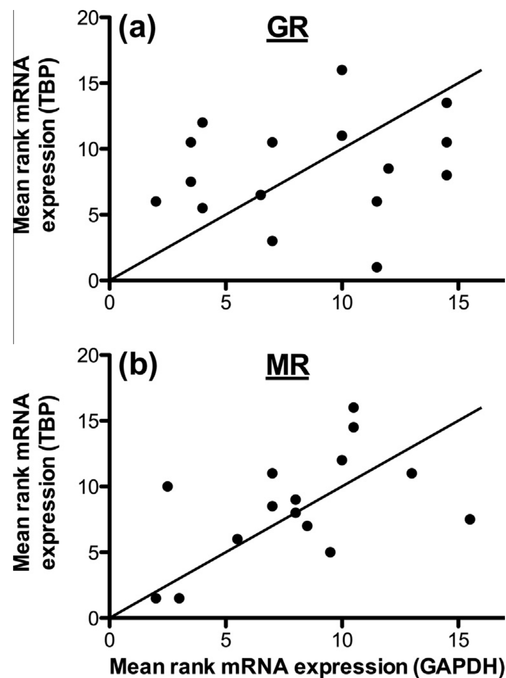

Fig. 7. Rank-order correlations between two different reference genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and TATA-box binding protein (TBP) for glucocorticoid receptor (GR, panel a) and mineralocorticoid receptor (MR, panel b) mRNA expression in house sparrow brains (n = 16). Birds are placed on the x-axis based on mean GAPDH mRNA expression rank. Birds are placed on the y-axis based on mean rank of TBP mRNA expression.

only partial coding sequences were available for house sparrow MR, GR, GAPDH, and TBP. The lack of genomic sequence data makes it more difficult to ensure that the customized specific primers were truly specific to the target gene of interest. This made gel electrophoresis analysis and sequencing of products crucial for confirming the specificity of our primer sets. Using DNA sequencing of purified PCR products, we were able to match at least 16 matching consecutive bases between sequenced product and target gene for all primers. The likelihood of a 16-base sequence occurring again at random is $1/2^n$ or 1 in 4.3×10^9 bases. The entire genome of the chicken is only 1.05×10^9 (International Chicken Genome Sequencing Consortium, 2004) and of the zebra finch is 1.23×10^9 (Warren et al., 2010); assuming a similar genomic length in house sparrows, this makes it highly unlikely that this exact sequence of bases would randomly appear again anywhere else in the genome. The genome is not a random assemblage of bases, of course, and some sequences are more likely to be repeated than others; however, there is no data on the likelihood of specific sequence repeats in the house sparrow genome.

A second limitation was that chemical adrenalectomy using mitotane was required for the receptor binding assay. This drastic change in circulating CORT is likely to cause major changes in mRNA and protein expression before tissue harvest. For example, only 3 days of treatment with RU486, a GR antagonist, resulted in a significant increase in GR mRNA and a decrease in GR protein in the brain and the liver of rainbow trout (Alderman et al., 2012). Unfortunately, the receptor binding assays require a steroid-free preparation to accurately determine the number of CORT binding sites (Breuner and Orchinik, 2009) and a method of rapidly removing endogenous CORT from receptors before perfusion remains undiscovered. Future studies could examine alternative techniques to quantify house sparrow GR and MR, such as in situ hybridization, immunohistochemistry and Western blotting.

Third, the physiological relevance of these results is limited. GR and MR are not evenly distributed throughout the brain, but rather show pockets of expression in certain brain nuclei such as the hippocampus (reviewed by Korte (2001)). The distribution of receptors is believed to play a major role in the physiological and behavioral changes they mediate. Homogenization of a highly complex tissue like brain, however, results in the averaging of these subpopulations of cells, and therefore cannot be used to explain direct neurological or behavioral effects in specific portions of the brain. The benefit of the present approach, however, is that both GR and MR radioligand binding and mRNA expression are consistent across the two brain halves (Figs. 3 and 4), allowing the two brain halves to be processed in the two different assays and yet be comparable.

Notwithstanding the above limitations, we found no evidence that mRNA and protein levels were correlated for either GR or MR. While mRNA may not be able to serve as a proxy for functional protein, an understanding of both receptor and transcript dynamics is crucial for a thorough understanding of stress physiology, as well as other biological systems (Hatzimanikatis and Lee, 1999). In fact, studies examining both protein and mRNA expression changes in the house sparrow could provide further insight into the mechanisms of HPA axis regulation. For example, in the laboratory rat, adrenalectomy increases both MR binding and mRNA in the hippocampus, suggesting an inhibitory role of glucocorticoids at the MR gene either through MR or GR action (Herman et al., 1999).

Our study also demonstrates that reference genes need to be chosen carefully and their use must be justified and validated for each experiment. GAPDH was initially chosen because of its widespread use as a reference gene for RT-qPCR experiments. GAPDH expression, however, can be highly variable across human tissues, and even within a single tissue type, although to a lesser degree (Barber et al., 2005). Furthermore, De Martino et al. (2004) found that GR physically interacts with the chicken ovalbumin upstream promoter transcription factor II, which has an important role in glucose metabolism, in which GAPDH also plays a major role. Therefore, future studies wishing to look at processes such as chronic stress, which can have major impacts on energy regulation, might seek a reference gene that is not so closely tied to glucose metabolism. TBP was subsequently chosen as a second reference gene because of its role as a general transcription factor, which is likely to be expressed ubiquitously. TBP has previously been used as a reference gene in house sparrows (Helfer et al., 2006). However, there could also be experimental conditions, such as tissue growth, under which TBP levels might change. Researchers opting to use RT-qPCR in any species would do well to validate the stability of their reference genes across treatment groups before attempting to quantify their gene of interest. The fact that these two reference genes produced very different rank-orders of mRNA expression (Fig. 7) indicates that the expression of one, or both, was not consistent across individuals.

In conclusion, GR and MR protein binding and mRNA expression were not correlated in neural tissue of house sparrows, at least on a whole-brain level. Furthermore, the lack of correlation between GR and MR protein and mRNA expression in other taxa and tissues (Alderman et al., 2012; Herman et al., 1999; Vijayan et al., 2003) suggests that there is a widespread difference in the regulation of mRNA transcription and translation for these receptors. While the quantification of mRNA expression provides valuable information at one level of receptor regulation, the quantification of functional protein using techniques such as radioligand binding assays provides data relevant to the immediate capability of the animal to respond to a stress-induced increase in CORT. Hopefully, future

studies will better incorporate several different tools for examining the various levels of HPA axis regulation.

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