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Locally elevated cortisol in lymphoid organs of the developing zebra finch but not Japanese quail or chicken

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ABSTRACT

Glucocorticoids are important for production of functional lymphocytes and immunity. In altricial neonates, adrenal glands are unresponsive and local glucocorticoid synthesis in lymphoid organs may be necessary to support lymphocyte development. Precocial neonates, in contrast, have fully responsive adrenal glucocorticoid production, and lymphoid glucocorticoid synthesis may not be necessary. Here, we found that in altricial zebra finch hatchlings, lymphoid organs had dramatically elevated endogenous glucocorticoid (and precursor) levels compared to levels in circulating blood. Furthermore, while avian adrenals produce corticosterone, finch lymphoid organs had much higher levels of cortisol, an unexpected glucocorticoid in birds. In contrast, precocial Japanese quail and chicken offspring did not have locally elevated lymphoid glucocorticoid levels, nor did their lymphoid organs contain high proportions of cortisol. These results show that lymphoid glucocorticoids differ in identity, concentration, and possibly source, in hatchlings of three different bird species. Locally-regulated glucocorticoids might have species-specific roles in immune development.

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1. Introduction

Animals exhibit dramatic variation along a spectrum of developmental strategies, ranging from undeveloped altricial offspring that are completely dependent on their parents, to developed and independent precocial offspring (Starck and Ricklefs, 1998). Avian and mammalian offspring rely heavily on innate immunity and maternal antibodies for protective immunity in early life, because lymphocytes that provide antigen-specific immunity are not yet mature and functional (birds, Davison et al., 2011; Klasing and Leshchinsky, 1999; mammals, Levy, 2007; Pilorz et al., 2005). A large proportion of lymphocytes are produced during early life (Adkins et al., 2004; Davison et al., 2011), creating a critical period during which environmental conditions can have long-term programming effects on adaptive immunity (Hodgson and Coe, 2006). The mechanisms underlying this developmental programming, however, are largely unknown.

Glucocorticoids (GCs) are steroid hormones (Fig. 1) that mediate environmental effects on immunity (Martin, 2009) and are critical for the production of functional T lymphocytes that are sufficiently responsive to foreign antigens (Mittelstadt et al., 2012). Glucocorticoids antagonize signaling of the T cell antigen receptor (Vacchio et al., 1994; Jamieson and Yamamoto, 2000; Van Laethem et al., 2001), and thus promote the survival of developing lymphocytes with higher affinity for self peptide in the context of major histocompatibility complex (MHC) molecules (Ashwell et al., 2000). Circulating GCs in the blood may not be sufficient for development

Abbreviations: GC, glucocorticoid; GR, glucocorticoid receptor; HAW, Hawaiian strain of Japanese quail; HPLC, high-performance liquid chromatography; MR, mineralocorticoid receptor; P0, posthatch day 0; PCR, polymerase chain reaction; RIA, radioimmunoassay; SEM, standard error of the mean; SHRP, stress hyporesponsive period; SPE, solid-phase extraction.

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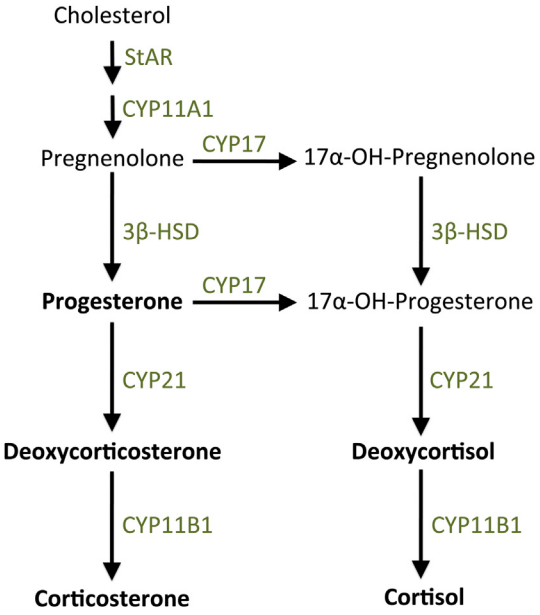

Fig. 1. Simplified glucocorticoid-synthetic pathway. Steroids quantified in this study are shown in bold, and steroidogenic enzymes are shaded in green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of competent lymphocytes, as mouse pups and songbird nestlings (like altricial neonates of other species) experience a stress hyporesponsive period (SHRP) during which circulating GCs are extremely low and unresponsive (Schmidt et al., 2003; Wada, 2008). However, murine lymphoid organs synthesize GCs (Lechner et al., 2000; Pazirandeh et al., 1999; Vacchio et al., 1994), resulting in locally elevated GC levels in these organs (Taves et al., 2015). Interestingly, murine lymphoid organs can have high levels of cortisol, as well as the predominant circulating murine GC, corticosterone (Taves et al., 2015). High cortisol levels are also seen in lymphoid organs (thymus, bursa of Fabricius, and spleen) of an altricial bird, the zebra finch (Schmidt and Soma, 2008), where the predominant circulating avian GC is also corticosterone. Together, these findings suggest potentially distinct roles of cortisol and corticosterone in developing lymphoid organs.

Precocial offspring, in contrast, have responsive circulating GCs (Brown and Spencer, 2013; Starck and Ricklefs, 1998), which may be sufficient to support normal lymphocyte production. Circulating GCs of precocial offspring, like circulating GCs of adults, are highly responsive to environmental conditions. Chronically increased or decreased GC levels in the blood could alter lymphocyte development, resulting in lymphocytes with corresponding increased or decreased reactivity (here, we use "reactivity" to refer to the overall strength of antigen receptor signaling of the lymphocyte repertoire, and the resulting likelihood of recognizing and responding to a given antigen). In this way, changes in circulating GCs might be a mechanism by which environmental conditions program adaptive immunity. Alternatively, lymphoid organs of developing chickens appear to synthesize cortisol *in vitro* (Lechner et al., 2001), indicating that chicken offspring may also require locally-elevated GC levels for development of functional lymphocytes.

Here, we aimed to determine whether lymphoid GCs of three different avian species follow similar patterns of local elevation (relative to circulating GCs) during post-hatch immune development, or whether lymphoid GC levels instead follow distinct, species-specific patterns. We measured two endogenous GCs: corticosterone, the predominant circulating avian GC, and cortisol, a GC classically thought to be absent in birds, but which we have found in avian lymphoid organs (Schmidt and Soma, 2008). Locally elevated lymphoid GC levels (in lymphoid organs relative to circulating blood) across species would suggest that the maintenance of stable GC concentrations is critical for lymphocyte development. However, if local GC levels remain similar to circulating GC levels, this would suggest that environmental conditions play a stronger role in determining the GC levels to which lymphoid organs are exposed. Furthermore, we also quantified GC precursors, 11-deoxycorticosterone (deoxycorticosterone), 11-deoxycortisol (deoxycortisol), and progesterone. The presence of locally elevated GCs together with their precursors suggests that local synthesis from upstream precursors could play a major role in local GC elevation (Taves et al., 2011a, 2015). In addition, these steroids can independently bind to and regulate activity of steroid receptors (Gomez-Sanchez, 2014). Samples were collected from the Zebra finch *(Taeniopygia guttata)*, the Japanese quail *(Coturnix coturnix japonica)*, and the chicken *(Gallus gallus domesticus)*. Comparative studies of developmental strategies have largely focused on birds, providing substantial background information and thereby making them well suited to explore the role of steroids in immune development. Furthermore, the developmental effects of glucocorticoids have been extensively investigated in these three domestic species, making them especially useful models (Schoech et al., 2011).

We examined birds as hatchlings and as juveniles, as lymphocyte production is greatest in early life (Orkin and Zon, 2008) and lymphoid organs regress with age (Davison et al., 2011; Glick, 1956). For both quail and chicken we included two strains, to look for differences both within species and across species. Because lymphocyte selection is extremely stringent to achieve immunocompetence while avoiding autoimmunity, we hypothesized that offspring of all three bird species would have locally elevated GCs and GC precursors in developing lymphoid organs relative to levels in circulating blood.

2. Materials and methods

2.1. Subjects

Zebra finches are born naked, immobile, with closed eyes, and with highly immature tissues, and parents provide food, warmth, and protection against predators and parasites (Starck and Ricklefs, 1998; Zann and Bamford, 1996). Altricial development (perhaps in part due to the stress hyporesponsive period) facilitates rapid posthatch growth (Ricklefs, 1979; Wada, 2008; Wada et al., 2009). Due to immature sensory systems (Herrmann and Bischof, 1988), immature physiological responses to stimuli (Wada et al., 2009) and buffering of environmental conditions by parents (Lindström, 1999), altricial hatchlings have limited interactions with environments beyond the nest. Parents even engage in behaviors to protect the nest from parasites (Petit et al., 2002).

Zebra finches in this study were from a captive colony maintained at the Advanced Facility for Avian Research at the University of Western Ontario. Finches were housed on a 14 h: 10 h light:dark cycle, and had ad libitum access to grit, cuttlefish bone, water, and seed (11% protein, 6% lipid; Living World premium finch seed). Breeding pairs were given a nest box, and received daily supplements of hardboiled eggs, cornmeal, and bread. Hatchling finches were collected from nest boxes on the day of hatch (P0, or posthatch day 0), and juveniles (still housed with parents) were collected at P30, which is approximately one-third of the age of sexual maturity (gonads mature at approximately P90).

Japanese quail and chickens are born feathered, mobile, with open eyes, and able to actively forage for themselves (McNabb and McNabb, 1977; Nichelmann and Tzschentke, 2002; Ottinger, 2001).

These functional physiological systems come at the cost of reduced post-hatch growth rates (Ricklefs, 1979). Precocial offspring experience little or no post-hatch stress hyporesponsive period; they have a functioning GC response and they are responsive to environmental stimuli (Brown and Spencer, 2013; Freeman and Manning, 1984). While they may still be led to food, brooded, and protected by their parents, these offspring are immediately exposed to environmental conditions beyond the nest that impact their survival.

Japanese quail in this study were from pure lines maintained in a UBC animal facility at the Agassiz Pacific Agri-Food Research Centre (Silversides et al., 2013). We looked at two strains of Japanese quail: the first strain (UBC line) is a long-domesticated line closed in 1968, while the second strain was derived from feral birds captured in Hawaii in 1981 (HAW line). Quail were housed on a 12 h: 12 h light:dark cycle, and received a nutritionally complete diet containing 2900 kcal/kg metabolizable energy and 24% crude protein and water to allow for ad libitum consumption. Hatchling (P0) quail were collected from a 37 °C incubator, and group-housed juveniles were kept in a 33 °C brooder and collected at P14, which is approximately one-third of the age of sexual maturity.

Chickens in this study were from pure lines maintained at the same facility at the Pacific Agri-Food Research Centre (Silversides et al., 2007). We looked at two breeds of chickens both bred for egg laying, the White Leghorn and the Rhode Island Red. These breeds have been previously used to investigate bursa of Fabricius (hereafter bursa) development; the bursa is larger at hatch and grows faster in the While Leghorn than the Rhode Island Red (Glick, 1956). Chickens were housed on a 12 h: 12 h light:dark cycle, and received a nutritionally complete diet containing 2800 kcal/kg and 18.5% crude protein and water to allow for ad libitum consumption. Hatchling (P0) chicks were collected from a 37 °C incubator, and group-housed juveniles were kept in a 33 °C brooder and collected at P42, which is approximately one-third of the age of sexual maturity.

For all three species, no subject was ever housed in isolation, and no subjects received antibiotics or vaccines. Egg incubation periods for these species are 12–15 d (zebra finch), 17–18 d (Japanese quail), and 21 d (chicken). Sex was determined by visual inspection of the gonads except for hatchling finches, in which case sex was determined using PCR as previously described (Soderstrom et al., 2007). The number of subjects collected for each species, strain, and sex is given in Table 1. Protocols followed approved institutional guidelines (UBC protocol A09-0395, UWO protocol 2007-089, and Agassiz Research Centre protocol P1101), and were in compliance with regulations established by the Canadian Council on Animal Care.

Table 1
Number of subjects of each species, strain, and sex.

Species	Strain	Sex	Hatchling	Juvenile
Zebra finch	na	Female	6	3
		Male	10	5
Japanese quail	UBC	Female	8	5
		Male	0	4
	Hawaiian (HAW)	Female	4	5
		Male	4	4
Chicken	White Leghorn	Female	3	3
		Male	6	5
	Rhode Island Red	Female	5	2
		Male	4	6

Note: For zebra finch hatchlings only, tissues from two subjects were pooled, resulting in the number of analyzed samples being half of the number of subjects collected (i.e., 3 female sample pools and 5 male sample pools for each tissue).
na = not applicable.

2.2. Tissue collection

Finch hatchling blood samples were collected by cardiac puncture within 3 min of researchers approaching the cage, and blood was stored on wet ice. Brachial blood samples from P30 finches were also collected within 3 min, into two tubes, one kept as whole blood and the other centrifuged for separation of plasma. Due to small sample volume, only whole blood was collected from P0 finches to optimize the chance of steroid detection. Immediately after blood collection, subjects were sacrificed by rapid decapitation and tissues (thymus, bursa of Fabricius, and spleen) were dissected and frozen on dry ice. Thymus was not collected from P0 finches due to its small size. All samples (plasma, whole blood, and lymphoid organs) were stored at −80 °C.

For quail and chickens, subjects were sacrificed by rapid decapitation, trunk blood was collected into two tubes within 3 min of researchers approaching the cage, and blood was stored on wet ice. One tube was kept as whole blood, and the other centrifuged for separation of plasma. Immediately after blood collection, tissues (thymus, bursa, and spleen) were dissected and frozen on dry ice. All samples were stored at −80 °C.

2.3. Steroid extraction

Total steroids were extracted from all samples using solid-phase extraction (SPE) with C_{18} cartridges (Newman et al., 2008; Taves et al., 2010, 2015). Briefly, samples were measured to the closest μl (plasma, blood) or the closest 0.1 mg (thymus, bursa, spleen), and transferred into a chilled 5-ml tube containing five 2.3 mm ceramic beads. The maximum tissue sample size was 250 μl or mg; larger tissues were bisected until a portion under this size was obtained. All samples were then diluted with 19 volumes of 84% methanol and homogenized using an Omni Bead Ruptor. Homogenates were incubated overnight at 4 °C, and the following day supernatants (up to 1 ml) were diluted with 10 ml water. Cartridges (Agilent #12113045) were primed with 3 ml methanol, equilibrated with 10 ml water, and then samples were loaded. Samples were washed with 10 ml 40% methanol, and steroids eluted with 5 ml 90% methanol (Taves et al., 2010). All reagents were HPLC-grade. Eluates were dried in a vacuum centrifuge (ThermoElectron SPD111V) at 60 °C.

2.4. Steroid separation

Steroids were then separated using reverse-phase high performance liquid chromatography (HPLC, Gilson 322), as previously described (Taves et al., 2015). Briefly, dried steroid residues were resuspended in Solvent A (30% acetonitrile, 0.01% formic acid), injected onto a Waters SymmetryShield C_{18} column (4.6 × 250 mm), and eluted at a flow rate of 1.0 ml/min over 45 min. Elution began with 100% Solvent A, with a linear increase to 100% Solvent B (100% acetonitrile, 0.01% formic acid) from 20 to 45 min. This run time resulted in clean separation of our steroids of interest, which were collected in 3-min fractions. Other steroids did not co-elute with our steroids of interest, with the exception of cortisone, which co-eluted in the cortisol fraction (Taves et al., 2015). It is unlikely that this affected our cortisol measurements, as we measured cortisol with an antibody that has minimal cross-reactivity with cortisone (0.13%, Supplementary Table 1), and as this protocol has been validated for identification of cortisol using liquid chromatography-tandem mass spectrometry (Taves et al., 2015).

2.5. Steroid immunoassays

Steroids (Fig. 1) were measured in duplicate using specific and sensitive immunoassays, as previously described (Taves et al., 2015). Briefly, progesterone, corticosterone, and cortisol were quantified using commercial immunoassay kits (Supplementary Table 1), and deoxycorticosterone and deoxycortisol were quantified by radioimmunoassay (RIA) using commercially available antibodies (Supplementary Table 1), with charcoal-dextran separation of antibody-bound and free steroids prior to counting in a liquid scintillation counter. For each steroid, recovery was determined by spiking known steroid amounts into tissue samples, and comparing these with unspiked samples from the same tissue pools (Taves et al., 2011b).

2.6. Statistical analysis

Nondetectable steroid samples (those below the lowest standard on the standard curve or those lower than the average water blank + two standard deviations, whichever was greater) were set to zero. Log-transformed data were analyzed using linear mixed-effects models in (R Core Team, 2005) and Prism 5, with tissue type (e.g. whole blood, bursa) as a within-subjects factor and with sex, age, and strain as between-subjects factors. Planned comparisons were conducted using paired t-tests. Total organ steroid levels were compared with whole blood steroid levels (Taves et al., 2010, 2011b), as whole blood can be more reflective of total circulating steroid levels than plasma, and both unbound plasma steroids and erythrocyte-associated steroids are available to enter other tissues (Hiramatsu and Nisula, 1987, 1991). Plasma steroids were nonetheless quantified to allow comparison with other published data (Supplementary Table 2), and patterns were similar in plasma and whole blood. Significance was set at $\alpha = 0.05$, and data are shown as mean ± SEM.

3. Results

We examined subjects at two developmental timepoints, as hatchlings and as juveniles at one third of the age at sexual maturity. Absolute and relative body masses are given in Supplementary Table 3 and Supplementary Figure 1a. To look for evidence of tissue-specific GC regulation, we compared concentrations of steroids in lymphoid organs with those in circulating blood. Locally elevated GCs, especially together with locally elevated precursors, are consistent with local GC synthesis (Taves et al., 2011a, 2015).

3.1. Zebra finch steroids

In the hatchling zebra finch, all steroids were at low concentrations in circulating blood, with corticosterone present at higher concentrations than other steroids. In contrast, the hatchling bursa and spleen had highly elevated cortisol levels, with cortisol levels 30- and 35-fold higher, respectively, than blood cortisol levels. In contrast, corticosterone, the expected avian GC, was not locally elevated (Fig. 2d,e, Table 2). Within lymphoid organs, cortisol was the predominant GC, with concentrations nearly an order of magnitude higher than corticosterone concentrations, whereas cortisol and corticosterone concentrations were both low in the blood (Table 3). Hatchling lymphoid organs also had locally elevated progesterone and deoxycorticosterone (Fig. 2a,b, Table 2). The hatchling spleen had near-identical patterns to the hatchling bursa, with locally elevated cortisol but not corticosterone, and cortisol as the predominant GC (Fig. 2d,e, Table 2, Table 3). Progesterone and deoxycorticosterone were also similarly elevated, although deoxycortisol was not (Fig. 2a,b, Table 2). Patterns were similar in female and male hatchlings. Thymus could not be collected from hatchling finches, due to its small size.

In juvenile zebra finches, all steroids remained low in circulating blood. As in hatchlings, lymphoid organs (thymus and bursa) had locally elevated cortisol levels, with cortisol levels approximately 4-fold higher than blood cortisol levels. Corticosterone was not locally elevated (Fig. 2d,e, Table 2). In thymus and spleen, cortisol concentrations were between 4- and 5-fold higher than corticosterone concentrations, while in blood cortisol concentrations were far less than half of corticosterone concentrations (Table 3). Juvenile thymus also had locally elevated progesterone and deoxycortisol (Fig. 2a,c; Table 1). Patterns were similar between female and male juveniles.

3.2. Japanese quail steroids

In hatchling Japanese quail, corticosterone was present at higher concentrations than other steroids in circulating blood, and steroid levels were similar between UBC and HAW strain hatchlings. Hatchling lymphoid organs did not have locally elevated levels of either cortisol or corticosterone (Fig. 3d,e), and cortisol concentrations were similar to or lower than corticosterone concentrations in all tissues (Table 3). However, all hatchling lymphoid organs had locally elevated levels of the GC precursors: progesterone, deoxycorticosterone, and deoxycortisol (Fig. 3a–c, Table 2). These were especially high in the spleen.

In juvenile quail, circulating steroid patterns were similar in UBC and HAW strains. However, there were strain-specific patterns in local GC levels. Corticosterone was locally elevated in all lymphoid organs of UBC but not HAW quail juveniles (Table 2), while cortisol was similarly elevated in spleen regardless of strain (Fig. 3e). Corticosterone was higher than cortisol, and the ratio of these steroids was the same across compartments. Juveniles however had limited, and strain-specific, elevation of GC precursors, with elevated deoxycorticosterone in the bursa (UBC only) and spleen (HAW only), and deoxycortisol in the bursa of both strains (Table 2, Fig. 3b,c). Progesterone was not elevated in any juvenile tissue (Fig. 3a).

3.3. Chicken steroids

In hatchling chickens, corticosterone was present at higher concentrations than other steroids in the blood, and circulating steroid levels were similar between White Leghorn and Rhode Island Red strains. Hatchling lymphoid organs did not have locally elevated levels of cortisol or corticosterone (Fig. 4d,e), and corticosterone was clearly the predominant GC in hatchling lymphoid organs (Table 3). Lymphoid deoxycorticosterone and deoxycortisol were also not locally elevated (Fig. 4b,c). Progesterone alone was locally elevated in all hatchling lymphoid organs (Fig. 4a).

In juvenile chickens, the bursa and spleen had locally elevated corticosterone, with levels slightly higher than levels in the blood (Table 2), and higher than levels of cortisol (Table 2). The thymus, in contrast, had locally elevated deoxycortisol (Fig. 4c). Progesterone had sex-specific patterns, with locally elevated concentrations in juvenile male but not female chickens (Table 2). Interestingly, the relative size of lymphoid organs was larger in hatchling and juvenile chickens than in zebra finches and quail (Supplementary Figure 1b,c).

4. Discussion

Here, we compared steroid profiles in hatchling and juvenile birds from three different avian species, and found species-specific

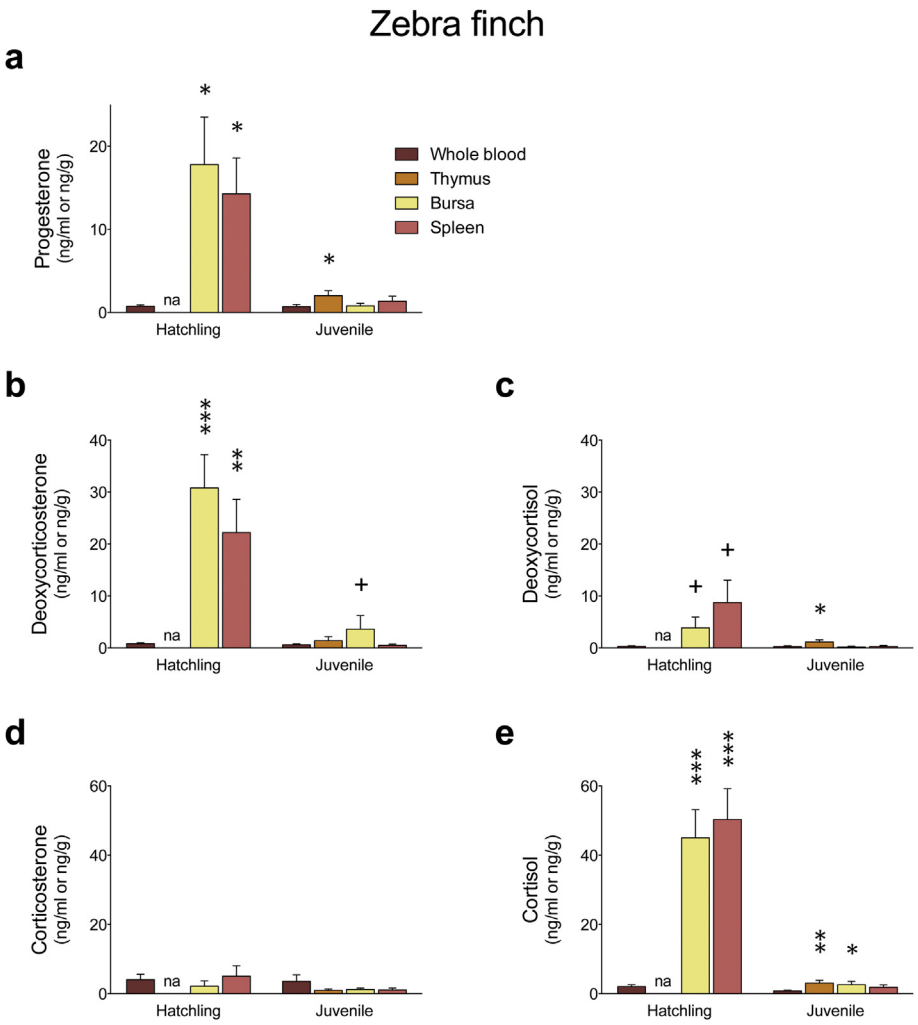

Fig. 2. Steroid levels in blood and immune organs of hatchling and juvenile zebra finches. a) Progesterone, b) deoxycorticosterone, c) deoxycortisol, d) corticosterone, and e) cortisol concentrations in whole blood (ng/ml), thymus, bursa of Fabricius, and spleen (ng/g) of zebra finch hatchlings (posthatch day 0) and juveniles (posthatch day 30). Thymus was not collected from hatchlings (na = not applicable). Statistically significant differences between tissue steroid levels and blood steroid levels are indicated as follows, + $p < 0.10$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Table 2
Relative steroid concentrations in lymphoid organs compared to blood. Ratios are expressed as a fraction (organ steroid concentration/blood steroid concentration).

Species	Strain	Sex	Steroid	Hatchling			Juvenile		
				Thymus	Bursa	Spleen	Thymus	Bursa	Spleen
Zebra finch			Progesterone	na	**19 ± 5**	**16 ± 5**	**2.2 ± 0.6**	1.2 ± 0.2	1.7 ± 0.7
			Deoxycorticosterone	na	**51 ± 20**	**28 ± 13**	2.3 ± 1.0	3.4 ± 1.7	1.0 ± 0.4
			Corticosterone	na	0.6 ± 0.6	0.9 ± 0.5	0.5 ± 0.2	0.7 ± 0.3	0.6 ± 0.4
			Deoxycortisol	na	5.1 ± 3.0	10 ± 6	**2.7 ± 0.9**	0.8 ± 0.3	0.7 ± 0.3
			Cortisol	na	**30** ± 9	**35** ± 9	3.9 ± **1.2**	**4.3 ± 1.9**	3.2 ± 1.7
Japanese quail			Progesterone	**8.9 ± 4.0**	**21 ± 12**	**49 ± 24**	1.1 ± 0.3	11 ± 10	5.0 ± 3.4
	UBC		Deoxycorticosterone	**5.2 ± 1.8**	**7.4 ± 2.9**	**12 ± 5**	1.1 ± 0.2	**1.5 ± 0.5**	2.7 ± 1.5
	HAW						0.8 ± 0.1	1.0 ± 0.4	**3.2 ± 1.0**
	UBC		Corticosterone	0.5 ± 0.1	0.7 ± 0.2	0.8 ± 0.2	**2.6 ± 0.7**	**7.1 ± 3.7**	**3.4 ± 1.0**
	HAW						1.0 ± 0.2	0.8 ± 0.2	1.3 ± 0.7
			Deoxycortisol	**17 ± 7**	**13 ± 4**	**29 ± 10**	0.8 ± 0.1	**1.5 ± 0.4**	1.2 ± 0.3
			Cortisol	0.8 ± 0.2	1.2 ± 0.3	2.2 ± 1.4	0.9 ± 0.1	1.2 ± 0.3	**2.7 ± 1.1**
Chicken		F	Progesterone	**1.7 ± 0.2**	**3.6 ± 0.3**	**4.7 ± 0.5**	0.8 ± 0.8	1.8 ± 1.8	0.7 ± 0.7
		M					**1.3 ± 0.1**	**2.6 ± 0.3**	**1.2 ± 0.1**
			Deoxycorticosterone	0.7 ± 0.2	1.0 ± 0.3	1.0 ± 0.6	1.0 ± 0.4	1.1 ± 0.5	0.9 ± 0.4
			Corticosterone	0.8 ± 0.1	0.9 ± 0.2	1.0 ± 0.2	1.1 ± 0.2	**1.8 ± 0.4**	**2.2 ± 0.5**
			Deoxycortisol	0.9 ± 0.1	1.3 ± 0.3	1.6 ± 0.4	**1.2 ± 0.2**	1.1 ± 0.2	1.0 ± 0.1
			Cortisol	0.8 ± 0.1	0.9 ± 0.1	1.2 ± 0.3	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.1

Bolded values indicate tissue steroid concentrations significantly higher than blood steroid concentrations (e.g., values significantly greater than 1; $p < 0.05$). Strain or sex are only listed separately when steroid ratios differ between strains or between sexes. Cases where tissue steroids were detectable and blood steroids were non-detectable were not included when calculating means. na = not applicable.

Table 3
Relative cortisol:corticosterone concentrations in blood and lymphoid organs of hatchling and juvenile finches, quail, and chickens. na = not applicable.

Species	Hatchling				Juvenile			
	Blood	Thymus	Bursa	Spleen	Blood	Thymus	Bursa	Spleen
Zebra finch	1.2 ± 0.4	na	9.2 ± 0.6	8.8 ± 1.1	0.4 ± 0.1	4.8 ± 1.5	3.2 ± 1.5	4.6 ± 1.6
Japanese quail	0.1 ± 0.1	1.1 ± 0.8	0.5 ± 0.2	1.0 ± 0.6	0.1 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	2.6 ± 1.5
Chicken	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.3	0.7 ± 0.4	0.7 ± 0.3	0.4 ± 0.3	0.3 ± 0.1

patterns of local steroid regulation in lymphoid organs. Circulating GCs were similar in zebra finch hatchlings and juveniles, while circulating GCs were higher in Japanese quail and chicken hatchlings than in juveniles. In contrast, lymphoid organs of finches had dramatic local elevation of GCs and GC precursors (compared to levels in blood), while lymphoid organs of quail and chickens only had locally elevated GC precursors.

4.1. The predominant lymphoid GC is species-specific

Our data provide strong evidence for local GC regulation in lymphoid organs of avian species and confirms the presence of cortisol in birds. Previous measurements of cortisol in zebra finches used immunoassay after SPE (Schmidt et al., 2009; Schmidt and Soma, 2008), and here we obtained very similar cortisol measurements after additional HPLC cortisol separation. Notably, while the major circulating GC in birds is corticosterone, the predominant lymphoid GC differed across the species examined: cortisol was high in the zebra finch, while corticosterone was high in the quail and chicken. In zebra finches, the presence of distinct systemic and lymphoid GCs suggests distinct mechanisms of action. Corticosterone binds to both the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) in zebra finch thymus and bursa cytosol, while cortisol binds only to GR (Schmidt et al., 2010). As MR and GR have distinct and sometimes opposing activities (Chantong et al., 2012; Ehrchen et al., 2007; Usher et al., 2010), variations in

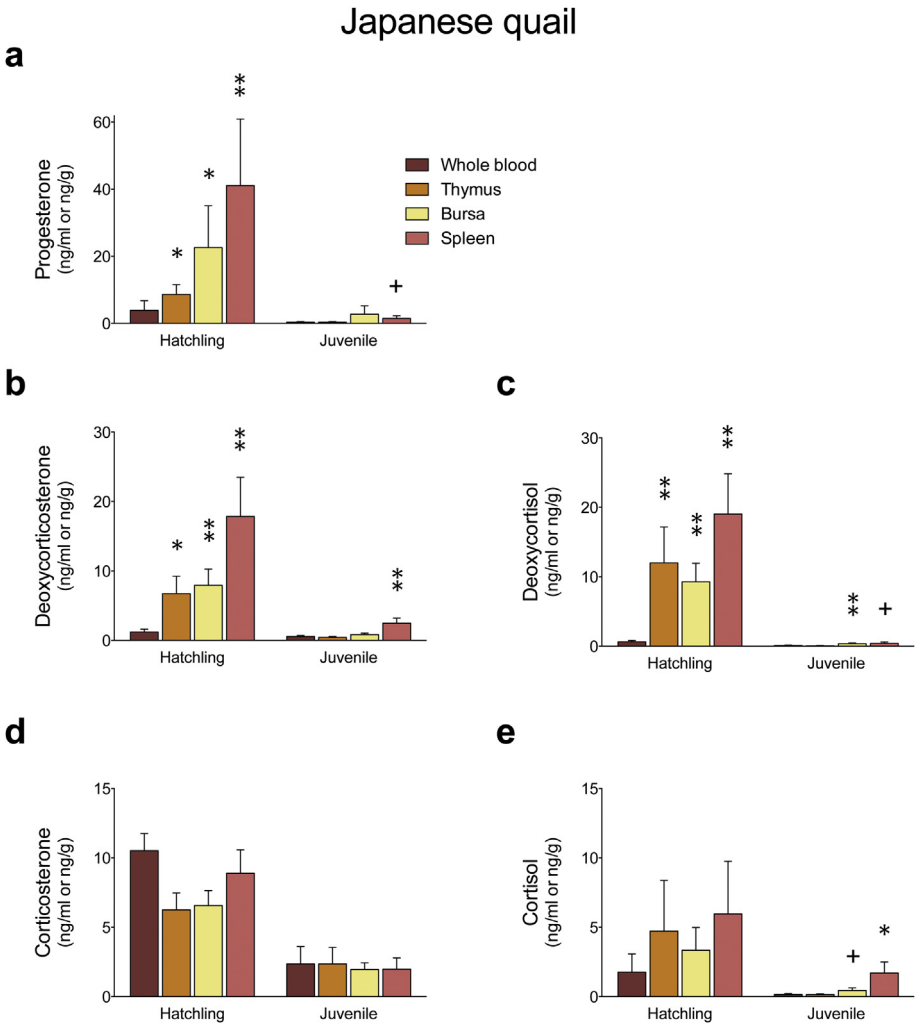

Fig. 3. Steroid levels in blood and immune organs of hatchling and juvenile Japanese quail. a) Progesterone, b) deoxycorticosterone, c) deoxycortisol, d) corticosterone, and e) cortisol concentrations in whole blood (ng/ml), thymus, bursa of Fabricius, and spleen (ng/g) of Japanese quail hatchlings (posthatch day 0) and juveniles (posthatch day 14). Statistically significant differences between tissue steroid levels and blood steroid levels are indicated as follows, + $p < 0.10$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

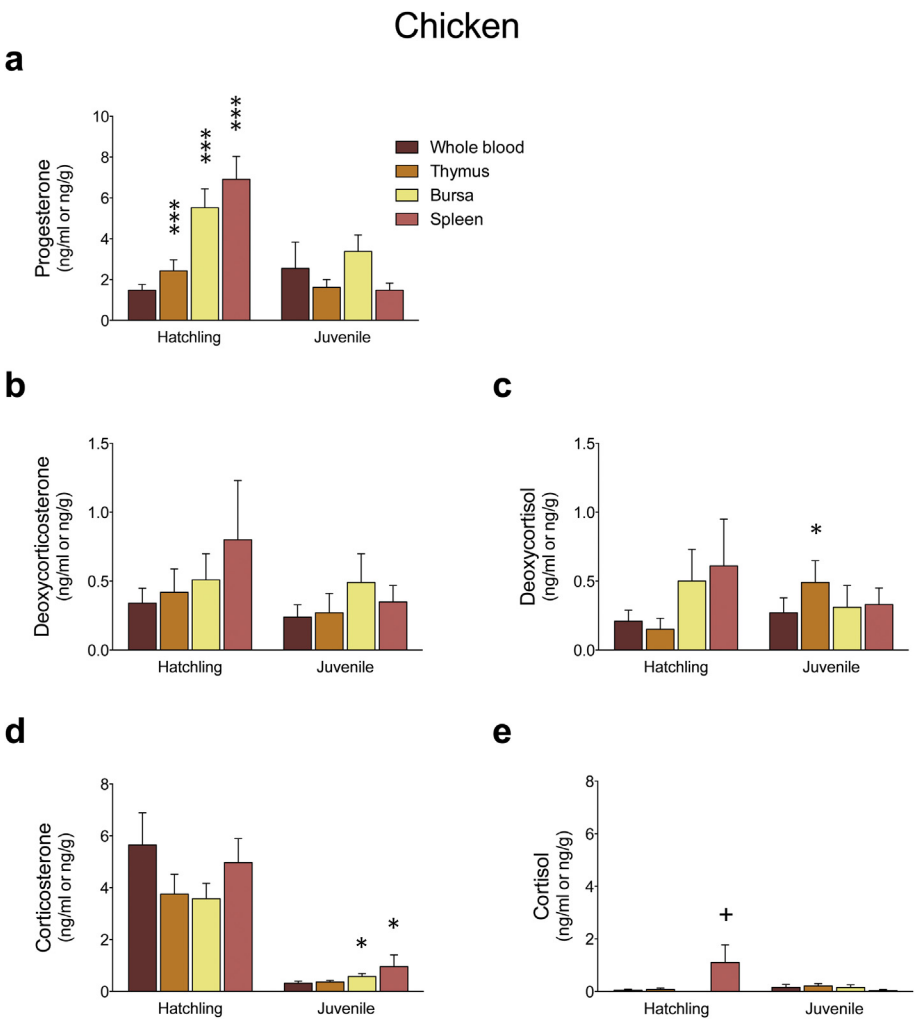

Fig. 4. Steroid levels in blood and immune organs of hatchling and juvenile chickens. a) Progesterone, b) deoxycorticosterone, c) deoxycortisol, d) corticosterone, and e) cortisol concentrations in whole blood (ng/ml), thymus, bursa of Fabricius, and spleen (ng/g) of chicken hatchlings (posthatch day 0) and juveniles (posthatch day 42). Statistically significant differences between tissue steroid levels and blood steroid levels are indicated as follows, + $p < 0.10$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

cortisol and corticosterone could result in fundamentally different cellular responses. Furthermore, immune cells can contain membrane-associated GRs (Bartholome et al., 2004; Vernocchi et al., 2013), and cortisol but not corticosterone binds to GR-like membrane sites in the finch bursa (Schmidt et al., 2010). Thus, lymphoid cells may distinguish between locally-derived cortisol and systemically-derived corticosterone. Indeed, cortisol has much stronger pro-apoptotic effects on chicken lymphocytes (Compton et al., 1990).

Cortisol and corticosterone signaling may both occur in quail lymphoid organs, which may have both GCs. In the chicken, however, corticosterone levels in lymphoid organs are much higher than cortisol, which is present at very low levels, and the use of distinct signaling pathways appears less likely. This finding of minimal concentrations of cortisol *in vivo* contrasts findings of another study also using juvenile White Leghorn chickens, which found that lymphoid organs produced cortisol *in vitro* (Lechner et al., 2001). The reason for this difference is unclear, but endogenous GC production may be altered by various environmental factors, such as diet or microbial exposure (Taves, 2015).

In contrast to GCs, progesterone was locally elevated in hatchling lymphoid organs of all three species. Progesterone may function as a precursor for local GC synthesis in the finch and quail, but was also elevated in lymphoid organs of the chicken, where there appears to be little GC production. Progesterone could function as a distinct signal, binding progesterone receptors in avian and mammalian lymphoid organs (Pasanen et al., 1998; Pearce et al., 1983; Tibbetts et al., 1999). Local progesterone elevation in neonatal birds and mice (Taves et al., 2015) indicates a conserved function in lymphoid development. In the periphery, progesterone promotes lymphocyte differentiation toward regulatory (Lee et al., 2012) and humoral (Hughes et al., 2013; Miyaura and Iwata, 2002) phenotypes, facilitating tolerance and preventing autoimmunity. Progesterone also affects lymphocyte development, regulating production of different lymphocyte subsets (Leposavić et al., 2014) and probably attenuating cell-mediated immunity (Perišić et al., 2013). Since lymphocyte heterogeneity and tolerance are fundamental to immunity, locally elevated lymphoid progesterone levels may facilitate this across vertebrates. Furthermore, it may be the balance between GCs and progesterone that regulates immune reactivity and tolerance.

4.2. Evidence for lymphoid GC synthesis

Adrenal GCs circulate through the blood and diffuse into various tissues, and are thus expected to be present at similar or lower

concentrations in other tissues compared to circulating blood. In contrast, the presence of locally elevated GC levels in specific tissues (versus circulating blood) is consistent with tissue-specific regulation (Taves et al., 2011b; Schmidt et al., 2008). Furthermore, when GC precursors are also locally elevated, this could be consistent with local GC synthesis, rather than GC uptake from the blood (Taves et al., 2011a, 2015). Our findings indicate that zebra finches, Japanese quail, and chickens may fall along a gradient of local versus systemic GC regulation.

In the zebra finch hatchling, progesterone, deoxycorticosterone, and cortisol are all locally elevated in lymphoid organs, suggesting that lymphoid cortisol might be synthesized from upstream precursors. Local elevation of deoxycortisol was nonsignificant, but deoxycortisol levels across tissues paralleled cortisol patterns, consistent with this interpretation. The juvenile thymus (but not bursa or spleen) also had elevated progesterone, deoxycortisol, and cortisol, suggesting reduced local GC synthesis.

In the Japanese quail, lymphoid corticosterone may be largely derived from the blood, as circulating and lymphoid corticosterone levels are similar. However, the precursors progesterone, deoxycorticosterone, and deoxycortisol are locally elevated in lymphoid organs, consistent with local GC production. It is possible that local synthesis does occur, but at a relatively low level that is masked by circulating GCs. Alternatively, if Cyp11b1 (Fig. 1) is not expressed, this could mean that deoxycorticosterone and deoxycortisol are end products of local steroid synthesis and bind to steroid receptors.

In the chicken hatchling, only progesterone was locally elevated in lymphoid organs, suggesting that lymphoid corticosterone is largely derived from the blood. Interestingly, this also suggests that progesterone on its own may be an important local signal; this might also be the case in the quail and finch. In juveniles, lymphoid organs had elevated corticosterone but not upstream precursors. Our data appear to contrast with previous work in which juvenile While Leghorn lymphoid organ homogenates demonstrated the full range of enzyme activities needed to produce GCs (Lechner et al., 2001). Furthermore, Lechner et al. found that chicken lymphoid organs preferentially synthesized cortisol, rather than corticosterone. Our data are not strongly suggestive of local GC synthesis, especially of cortisol, but it may be that local cortisol synthesis occurs at a low level that we were unable to detect, or differs because of other unknown factors differing between our studies.

Together, these findings suggest that local GC synthesis by lymphoid organs is greater in lymphoid organs of altricial young compared to precocial young. In altricial young, local GC synthesis may be critical to maintain development of functional lymphocytes during the stress hyporesponsive period, when circulating GC levels are low. In contrast, local GC synthesis may not be as important in precocial young. However, since this study examined only one altricial and two precocial species, additional species need to be examined for this conclusion to be strongly supported.

4.3. Across species, lymphoid GC levels decrease with age

In all three species we examined, lymphoid GC (and progesterone) levels were higher in hatchlings than juveniles. This age-related decrease corresponds with previous findings in developing zebra finches (Schmidt et al., 2009; Schmidt and Soma, 2008) and mice (Vacchio et al., 1994; Taves et al., 2015). The reduction in GCs also parallels lymphocyte production, which is highest in early life and decreases with age as the lymphoid organs regress (Davison et al., 2011). Zebra finch GC patterns in particular are extremely similar to those of developing altricial mice (Taves et al., 2015), suggesting a similar GC role in selection of appropriately reactive lymphocytes. Both GC hormones (Denver, 2009) and adaptive immunity (Cooper and Alder, 2006) are conserved across vertebrates, therefore it is quite plausible that GCs function in a highly conserved manner during lymphocyte selection. While the timeline of lymphoid organ colonization and lymphocyte production likely differs somewhat between the species in this study, generation of a diverse lymphocyte repertoire (and therefore selection of these lymphocytes) almost certainly continues posthatch in each of these species (Klasing and Leshchinsky, 1999).

4.4. Lymphoid GCs may differentially regulate immunity across species

Differential regulation of GCs across bird species could result in differential plasticity of the developing immune system. In altricial finch hatchlings, where immune GCs appear to be locally synthesized, programming of immune reactivity likely follows local GC patterns, as circulating GCs are low and unresponsive (Wada et al., 2009). In precocial quail and chicken offspring, however, lymphoid GC levels are similar to circulating GCs, and environmentally-induced changes in circulating GCs (due to factors such as predation, food shortage, or high pathogen load) could potently affect immune development. Thus, our data raise the possibility that the posthatch environment may impact the development of immune reactivity to a greater extent in Japanese quail and chicken than in finch hatchlings.

However, the differences in lymphoid GCs could relate to factors other than the mode of development. For example, zebra finches, Japanese quail, and chickens have been domesticated for dramatically different lengths of time (zebra finches, ~200 years (Griffith and Buchanan, 2010); Japanese quail, ~1000 years (Mills et al., 1997); chickens, ~8000 years (Fumihito et al., 1996; West and Zhou, 1988)), and the disparate profiles of lymphoid GCs in developing finches, quail, and chickens may be the result of domestication, and its accompanying selection for resistance to specific pathogens rather than general resistance to all pathogens (Bishop et al., 2010; Chaves et al., 2010). Species differences in lymphoid GCs could also relate to differences in body size (Supplementary Table 3), growth rate (Supplementary Figure 1), differential investment in immunity (Supplementary Figure 1), differences in diet, or from phylogenetic effects unrelated to developmental mode. Examination of a greater number of species is required to better understand GC roles in altricial and precocial life histories.

5. Conclusions

Our data show that lymphoid GCs are differentially regulated in offspring of different bird species, with strong evidence for local cortisol production in the altricial finch, and systemic regulation of lymphoid corticosterone in the quail and chicken. This differential regulation may be an important determinant of immune reactivity, and therefore susceptibility to pathogens and parasites throughout life. Furthermore, as progesterone levels are locally elevated in lymphoid organs of all hatchlings, this steroid may play a fundamental, heretofore unrecognized role in lymphoid development. This contrasts with the traditional view of steroids as immunosuppressive, instead indicating multiple roles in the development of a functional immune system.

Author contributions

Conceived and designed the experiments: MDT, KLS, FS, KKS.
Performed the experiments: MDT, JAL, TR, KLS, BAS, CM, KKS.
Analyzed the data: MDT, JAL, TR, KKS.
Contributed reagents/materials/analysis tools: FS, KKS.

Wrote the paper: MDT, JAL, TR, KLS, BAS, CM, FS, KKS.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dci.2015.09.004.

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