


Maternal glucocorticoid levels affect sex allocation in black howler monkeys

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Introduction

In anisogamous species, natural selection is expected to favor a 1:1 sex ratio at birth when sons and daughters cost the same to produce (Fisher, 1930). However, when the fitness benefits accrued from the production of sons or daughters differ, parents should bias parental investment and/or sex ratios (Trivers & Willard, 1973). Skewed sex ratios at birth occur in all vertebrate classes and have been studied in detail in some orders, such as Artiodactyla, Primata, Rodentia and Passeriformes (e.g. Hewison & Gaillard, 1999; Brown, 2001; Schino, 2004; Sheldon & West, 2004; Bonier, Martin & Wingfield, 2007; Ryan *et al.*, 2012, 2014). Although the adaptive significance of skewed sex ratios at birth is still disputed (Clutton-Brock & Iason, 1986; West & Sheldon, 2002; Silk & Brown, 2004), there is consensus over the importance of understanding sex allocation strategies, as they influence many dimensions of animal reproduction, behavior and demography.

Attention has been recently drawn to the mechanisms allowing the adaptive adjustment of sex ratios at birth, resulting in growing evidence that variation in the uterine environment around the time of conception is associated with sex differential conceptus survival. Specifically, variation in intrauterine glucose and hormone levels has been proposed as mechanisms for sex allocation (Krackow, 1995; Cameron, 2004; James,

Abstract

When sons and daughters impart different fitness benefits, mothers should bias investment according to offspring sex, in some cases modifying offspring sex ratios. Sex allocation in monotocous organisms is expected to be costlier than for polytocous species, as it imposes a greater loss of reproductive effort in terms of mating and fertilization. Here, we show that variation in glucocorticoid concentrations around the time of conception predicts infant sex in a monotocous anthropoid, the black howler monkey (*Alouatta pigra*), suggesting that sex allocation is linked to a hormonal mechanism that affects early embryonic development. Glucocorticoids signal environmental conditions, and in our study, their variation was positively related to factors affecting infant survival. These results offer insight into sex allocation strategies in monotocous organisms.

2013). Rather than being mutually exclusive, these mechanisms could act synergistically. Specifically, the influence of hormones on conceptus survival is probably related to their role in regulating intrauterine glucose availability, which both *in vivo* and *in vitro*, has been found to affect embryogenesis (Gutierrez-Adan *et al.*, 2001; Larson *et al.*, 2001; Mercader *et al.*, 2001). The biological and molecular actions of glucocorticoid hormones (Norman & Litwack, 1997) suggest that they are particularly suited to signal environmental conditions that may be relevant to sex allocation (Bonier *et al.*, 2007). Indeed, glucocorticoid levels during peri-embryonic development co-vary with infant sex in a number of species, including humans, white-crowned sparrows, Richardson's ground squirrels and bridled nail-tail wallabies (Bonier *et al.*, 2007; Chason *et al.*, 2012; Ryan *et al.*, 2014; Moore, Hayward & Robert, 2015). It is less clear, however, which environmental factors glucocorticoids are signaling, as only one study to date has tested how an environmental stressor, population density, affects the relationship between glucocorticoids and sex allocation: in bridled nail-tail wallabies, mothers living under unnaturally high population densities (captive) have higher glucocorticoid levels and produce more sons than mothers at natural densities (Moore *et al.*, 2015).

Mechanisms allowing for the facultative adjustment of sex ratios are expected to be under strong selection pressure, as

failure to match infant sex with maternal investment potential and environmental conditions lead to reduced fitness (Trivers & Willard, 1973; Clark, 1978). This pressure should be particularly intense in organisms that produce a single offspring at a time. In contrast with polytocous animals, which may adjust offspring sex within litters or clutches (Bonier *et al.*, 2007; Cameron *et al.*, 2008; Ryan *et al.*, 2012), sex allocation in monotocous organisms is expected to be costlier, as it implies the complete loss of reproductive effort (e.g. mating, fertilization) in a given breeding season. Therefore, mechanisms that allow for early sex allocation through the signaling of environmental conditions should be found in monotocous animals. Here, we provide the first evidence supporting the existence of such a mechanism in a primate, by demonstrating that infant sex at birth in the black howler monkey (*Alouatta pigra*) depends on maternal fecal glucocorticoid metabolite concentrations (henceforth glucocorticoid levels) around the time of conception, which seem in turn to be signaling environmental conditions associated with living in either extensive or fragmented forests.

Materials and Methods

Sampling of births, behavior and glucocorticoids

Research on black howler monkey populations in Campeche (Mexico) commenced in 2006. Between 2006 and 2012, we censused 10 groups of black howler monkeys (five in extensive forests and five in forest fragments; Table 1) by visiting each group three times a week (a total of $\approx 11\,000$ sampling days) (Dias *et al.*, 2015a). We identified and counted all individuals in each visit, and sampled the following behaviors of adult females: (1) the proportion of time females spent traveling (i.e. moving to a new area or tree) during 1-hour focal animal samples (Altmann, 1974; Dunn *et al.*, 2013); (2) all occurrences of agonistic social interactions in which females were involved during each visit (Dunn *et al.*, 2013; Gómez-Espinosa *et al.*, 2014); and (3) time spent feeding during 1-hour focal animal samples from leaves, fruits and flowers, which are the main food items of howler monkeys (Dias &

Rangel-Negrín, 2015). We multiplied time spent eating each food item by feeding rates reported for black howler monkey foods (Amato & Garber, 2014) to obtain estimates of the amount of each food type ingested (in grams). We collected 542 hours of behavioral data, with a mean \pm SD of 15.5 ± 2.2 behavioral sampling hours per female/birth.

Each time a newborn was observed, we registered its sex and we counted 184 days (gestation length: Van Belle *et al.*, 2009) back from birthdate to calculate putative time of conception. Because on occasions a group was not observed for two consecutive days, we used a loose definition of time of conception: ± 5 days around the putative time of conception. Therefore, except for infant sex, we calculated all observational variables within this timeframe (Table 1). In anthropoids, increases in glucocorticoid levels during pregnancy resulting from fetal and placental activity only occur during late gestation (Pepe & Albrecht, 1995; Majzoub & Karalis, 1999). It is therefore reasonable to assume that glucocorticoid measurements ± 5 days around conception were solely associated with adrenal activity of the mother. We could match 35 births with behavioral and fecal samples around the time of conception.

Fecal sample collection and analysis

We collected fresh fecal samples uncontaminated by urine whenever they could be matched with individuals. We collected samples from the forest floor and deposited them in polyethylene bags labeled with the identity of each individual. We kept the bags in a cooler with frozen gel packs while in the field, and stored them at the end of the day in a freezer at -20°C until steroid extraction was performed. Samples were stored for 3 to 5 months until the extractions were conducted. The freezing procedure used for storing the samples has been reported to have a weak time-storage effect on fecal hormone metabolites (Khan *et al.*, 2002).

We extracted glucocorticoids following a modification of the method described in Wasser *et al.* (2000) for the extraction of steroids. Briefly, 0.6 g of homogenized, lyophilized and pulverized feces was shaken for 20 hours in 4.0-mL of analytical-grade methanol. Extracts were then centrifuged (460 g for

Table 1 Variables analyzed in this study

Variable	Definition
Infant sex	Sex at birth determined through direct observation of genitalia (N females = 16; N males = 19)
Glucocorticoids around conception	Mean glucocorticoid metabolite concentrations in feces of mothers ± 5 days around conception time. Conception time was calculated by subtracting 184 days (pregnancy duration) from birth date ($N = 177$ fecal samples, mean \pm SD samples per conception = 5.1 ± 0.7 , range = 4–6 samples).
Habitat type	Fragmented (area < 1 km ² ; mean \pm SD area = 0.3 ± 0.4 km ² , range = 0.01–0.86 km ²) or extensive forests (area > 1 km ² ; mean \pm SD area = 289.4 ± 620.9 km ² , range = 3–1400 km ²)
Leaf consumption	Percentage of consumed food weight dedicated to eating leaves ± 5 days around the conception time (mean \pm SD = $58.7 \pm 27.8\%$, range = 5.8–100%).
Travel time	Percentage of time budget dedicated to moving to a new tree ± 5 days around the conception time (mean \pm SD = $9.7 \pm 7.3\%$, range = 1.1–29.5%)
Rate of agonistic interactions	Frequency of participation in agonistic interactions (as actor or receiver) divided by the total number of observation hours ± 5 days around the conception time (mean \pm SD = 0.02 ± 0.04 interactions/hour, range = 0–0.18 interactions/hour)

30 min) and the supernatant was recovered. After complete evaporation of the solvent in a water bath at 60°C for 20 hours, pellets were reconstituted with 3-mL albumin buffer and used for the glucocorticoid assays.

Assays were conducted at the Instituto de Ciencias Médicas y Nutrición Salvador Zubirán, in Mexico City. Glucocorticoid levels were measured using a solid-phase chemiluminescent enzyme immunoassay assay (Cortisol Immulite, Siemens, CA, USA; sensitivity = 5.5 nmol L⁻¹; calibration range = 28–1380 nmol L⁻¹) and an Immulite 1000 analyzer (Siemens, CA, USA). Pooled fecal extracts, when added to the standard curve points, exhibited an accuracy of $R^2 = 0.98$ ($N = 5$, $P = 0.001$), and serial dilutions of a fecal pool from howler monkeys yielded results that ran parallel to the kit standards ($R^2 = 0.97$, $N = 5$, $P < 0.001$). Samples were run in the order in which they were collected in a total of five assays, with a new set of quality controls performed in each assay. Glucocorticoid intra-assay variation averaged 6.8% (fecal extract pool, $N = 6$). Inter-assay variation, estimated for the five assays from fecal pools with varying levels of cortisol, averaged 19.3% (low), 15.1% (medium) and 7.2% (high). The protocols that we used to extract and quantify glucocorticoid levels in feces had been previously validated to detect the activation of the HPA axis in response to stressful stimuli in mantled howler monkeys (Rangel-Negrín *et al.*, 2014a). All samples were run in duplicate, and glucocorticoid values are reported as ng g⁻¹ (dry feces).

Data analysis

Binary logistic regression, implemented with generalized linear mixed models (GLMM), was used to determine the effect of (1) fecal glucocorticoid metabolite concentrations around the time of conception (ng g⁻¹ dry feces) and (2) the interaction between glucocorticoid metabolite concentrations and habitat type (fragments vs. extensive forests) on infant sex at birth.

Previous research indicated that the glucocorticoid levels of howler monkeys could be affected by energetic and psychosocial stressors (Cristóbal-Azkarate *et al.*, 2007; Dunn *et al.*, 2013; Gómez-Espinosa *et al.*, 2014), as well as by living in either extensive or fragmented forests (Martínez-Mota *et al.*, 2007; Rangel-Negrín *et al.*, 2014b). We therefore modeled variation in maternal glucocorticoids as a function of habitat type (females in five groups in extensive vs. females in five groups in fragmented forests), the amount of time spent traveling (a proxy for energetic stress), and participation in agonistic social interactions (a proxy for psychosocial stress). Because howler monkeys are generalist herbivores (Dias & Rangel-Negrín, 2015), and dietary variation may affect the measurement of hormone metabolites excreted in feces (Goymann, 2012), we included the amount of leaves consumed by mothers around the time of conception in our model of glucocorticoid variation. An analysis of covariance, also implemented with GLMM, was used to assess the effects of habitat type, travel time, rate of agonistic interactions and leaf consumption on fecal glucocorticoid metabolite concentrations around the time of conception. As some females contributed more than one

birth to the dataset (one birth = 7 females; two births = 11 females; three births = two females), female identity was included in both analyses as a random factor. GLMMs were run in SPSS v.22 (IBM Corp., New York). Effect sizes were calculated for significant predictors with Cohen's d index.

Results

The relationship between glucocorticoid levels and infant sex

Mean (\pm SE) fecal glucocorticoid levels among the 20 female black howler monkeys that we studied was 253.1 ± 8.4 ng g⁻¹ (range = 201.1–324.1 ng g⁻¹) in the ± 5 days surrounding conception of a son ($N = 19$ births), approximately 72% higher than when conceiving a daughter (146.7 ± 7.9 ng g⁻¹; range = 98.1–199.2 ng g⁻¹, $N = 16$). Glucocorticoid levels around the time of conception significantly predicted infant sex (general linear mixed model [GLMM] $F_{1,30} = 2.7$, $P = 0.011$, 95% CI = -0.111–(-0.016); $d = 3.05$), such that the probability of giving birth to a son increased with increasing hormone concentrations (Fig. 1).

Predictors of glucocorticoid levels

Only habitat type had a significant effect on glucocorticoid levels of females around the time of conception (Table 2) such that glucocorticoids were approximately 54% higher for females living in fragments than for females in extensive forests ($d = 1.92$; mean \pm SE fragments = 249.8 ± 10.8 ng g⁻¹, range = 162.5–324.1 ng g⁻¹; extensive forests = 162.3 ± 10.9 ng g⁻¹, range = 98.1–274.1; Fig. 2). The effect of glucocorticoid levels on offspring sex was independent of habitat type (GLMM $t_{1,30} = 0.9$, $P = 0.394$, 95% CI = -0.008–

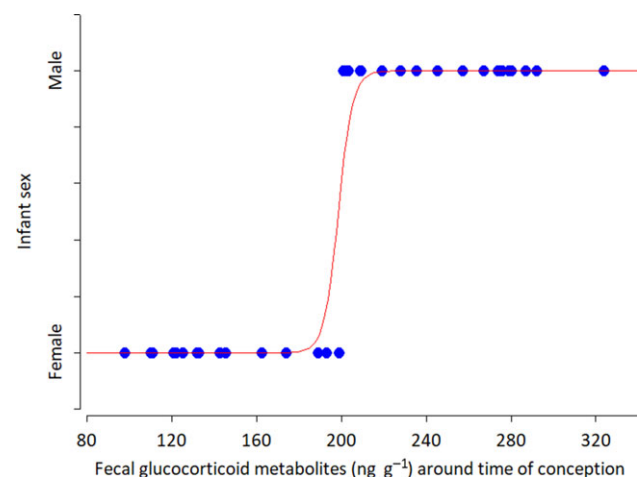
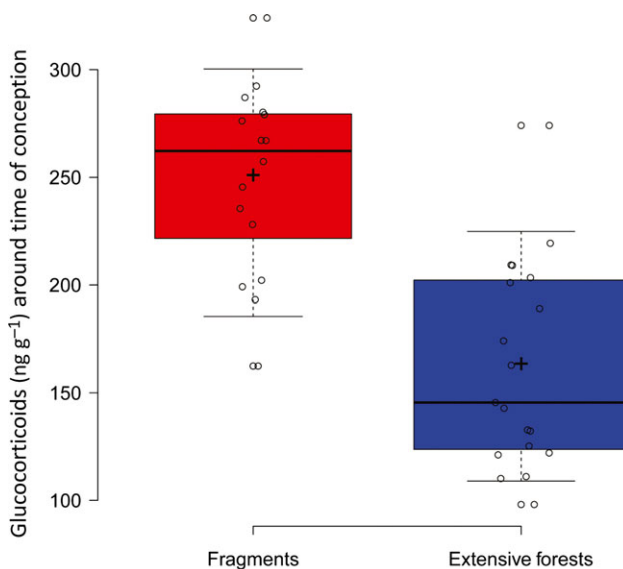


Figure 1 Sex allocation. The logistic regression detected a statistically significant effect of fecal glucocorticoid metabolite levels around conception time on infant sex at birth ($P < 0.001$, $N = 33$ births), with a d of 3.06.

Table 2 Generalized linear mixed model (GLMM) of variation in glucocorticoids of female black howler monkeys around conception time ($N = 35$ conceptions)

Factor	B	SE	t	P	95% C.I.	
					Lower	Upper
Habitat type	89.53	16.08	5.6	<0.001	56.70	122.5
Leaf consumption	0.484	0.30	1.6	0.117	-0.129	1.098
Travel time	-0.891	1.16	-0.8	0.447	-3.257	1.475
Agonistic interactions	130.8	201.7	0.7	0.522	-282.3	543.9

**Figure 2** Fecal glucocorticoid metabolites according to habitat type. Fecal glucocorticoid metabolites around conception time were significantly predicted by habitat type ($P < 0.001$) with a d of 1.92. Center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend to 5th and 95th percentile; crosses represent sample means; width of the boxes is proportional to the square root of the sample size; data points are plotted as open circles. N fragments = 16 conceptions, N extensive forests = 19 conceptions.

0.020), indicating that even in poor habitats, mothers with higher hormonal levels around conception were more likely to produce sons.

Discussion

Sex allocation studies have traditionally addressed two central topics, first the functional significance of producing infants of either sex, and second the mechanisms that allow for the facultative adjustment of infant sex; integrating both topics has been complicated. This is mostly because adaptive explanations are based on the premise that maternal condition and infant sex ratios co-vary, but the measurement of maternal condition has proven difficult, leading to inconclusive results (e.g. Silk & Brown, 2004). The recent demonstration that skewed sex ratios at birth result from sex differences in conceptus survival, which in turn, are dependent on attributes of the intrauterine

environment around conception, has narrowed the timeframe in which mechanisms for maternal adjustment of sex ratios should be measured. Furthermore, there is growing evidence that such mechanisms involve hormonal signaling of environmental conditions through glucocorticoids. At the outset of this study, however, comparative evidence of such mechanisms for monotocous animals was scarce (Chason *et al.*, 2012; Ryan *et al.*, 2012; Schwanz & Robert, 2014; Moore *et al.*, 2015). Our data reveal that the sex of black howler monkeys at birth is related to maternal glucocorticoids around the time of conception, as mothers gave birth to daughters only when their fecal glucocorticoid metabolites were below a threshold of *ca.* 200 ng g^{-1} (Fig. 3). In exploring which environmental cues glucocorticoids could be transducing into early embryonic development, we found that mothers living in disturbed forest fragments had higher glucocorticoid levels. Therefore, in this species, female conceptuses may be more vulnerable than males to the effects of glucocorticoid physiology, resulting in fewer female births under adverse environmental conditions.

It has been noted that different mechanisms for sex allocation may operate simultaneously, either synergistically or antagonistically (Cameron *et al.*, 2008; Navarra, 2010; Edwards, Cameron & Wapstra, 2016). Although it remains for future research to determine whether conceptus survival in black howler monkeys is directly determined by sex differential reactivity to glucocorticoids (Kraemer, 2000), or to some other physiological mechanism involving glucocorticoid secretion — either directly or as a byproduct (Ryan *et al.*, 2012; Moore *et al.*, 2015) — our results indicate that glucocorticoids may be signaling environmental conditions to females around the time of conception. Comparative data suggest that the monitoring of glucocorticoids *ca.* 5 days after the putative conception date could correspond to early embryonic development, most probably at the zygote-morulae-blastocyst stages (Hearn, Webley & Gidley-Baird, 1991). During these stages, differences in gene expression emerge between sexes (Wrenzycki *et al.*, 2002; Tan *et al.*, 2016), and it has been specifically proposed that overexpression of X-linked genes (e.g. G6PDH; Kobayashi *et al.*, 2006) may lead to higher mortality of female conceptuses (Gutierrez-Adan *et al.*, 2001). A mechanism enabling sex allocation at such an early stage of embryonic development as a function of environmental conditions is expected to be adaptive, because it minimizes maternal investment in offspring expected to provide limited fitness returns (Trivers & Willard, 1973). Primates have slow life histories compared to other mammals (Charnov & Berrigan, 1993), so late fetal or early infant mortality may impose very high costs

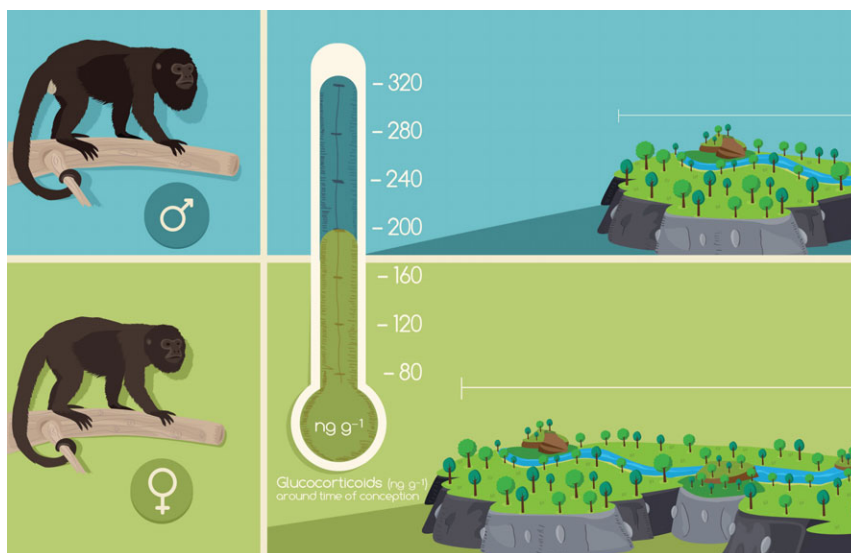


Figure 3 Sex allocation mechanism in black howler monkeys. The sex of black howler monkeys at birth is related to maternal glucocorticoids around the time of conception, as mothers gave birth to daughters only when their fecal glucocorticoid metabolites were below a threshold of ca. 200 ng g⁻¹. Mothers living in disturbed forest fragments had higher glucocorticoid levels.

in terms of time and energy to females. In black howler monkey females, for instance, the loss of a single infant leads to a 6.3% decrease in predicted lifetime reproductive success (Dias *et al.*, 2016). Therefore, mechanisms favoring early sex allocation are likely to have been under strong positive selection during primate evolution (Roberts *et al.*, 2012).

For social animals, the *local resource competition* hypothesis posits that, if there are sex differences in dispersal patterns, when resources are scarce mothers should produce more offspring of the dispersing sex in order to reduce future competition for resources with relatives (Clark, 1978). Therefore, four basic propositions need to be assessed to evaluate the local resource competition hypothesis: (1) dispersal patterns must vary between sexes; (2) resource availability must vary; (3) sex allocation depends on resource availability; (4) present and future resource availability are positively correlated. A number of implicit assumptions may be enunciated for this hypothesis, including immutability of dispersal patterns; and the expectation that when groups are not composed of a single matriline, all mothers respond to resource scarcity in the same manner, such that none expresses an alternative strategy of producing offspring from the philopatric sex to outcompete other matriline — as repeatedly observed in cercopithecine monkeys (e.g. Chapais, 1992). The premises underlying the local resource competition hypothesis have been tested, either directly or indirectly, and supported in several species (Johnson, 1988; Moore *et al.*, 2015). Although we currently lack data that allow testing all premises in black howler monkeys, we speculate that this hypothesis represents a viable explanation for sex allocation in this species, as: (1) female black howler monkeys may reside and reproduce in their natal groups, whereas males disperse to new areas (Van Belle *et al.*, 2012; Dias *et al.*, 2015a); (2) this species lives in forests that present temporal variation

in food availability (Valdez-Hernandez *et al.*, 2010; Schaffner *et al.*, 2012); (3) more daughters than sons are born in extensive than in fragmented forests, and resource availability is higher in the former than in the latter (Dias *et al.*, 2014, In press). We lack data, however, to assess whether resource availability around the time of conception reflects future resource availability (i.e. proposition 4).

The glucocorticoid levels of howler monkeys are generally higher in fragmented than in extensive forests as a consequence of low food availability, high anthropogenic pressures and both high feeding and reproductive competition (Cristóbal-Azkarate *et al.*, 2007; Martínez-Mota *et al.*, 2007; Dunn *et al.*, 2013; Gómez-Espinosa *et al.*, 2014; Rangel-Negrín *et al.*, 2014b). Therefore, glucocorticoid elevation in forest fragments signals a myriad of adverse environmental conditions that may have negative consequences for infant survival, and as a consequence, female reproductive success (Dias *et al.*, 2015a, 2016). Under such conditions, and according to the local resource competition hypothesis, the sex allocation mechanism described here allows females to produce more sons in forest fragments, which will not compete with their mothers because they will disperse. Furthermore, because maternal investment in daughters is higher than in sons, this mechanism allows females to reduce the burdens of rearing offspring when they produce sons (Dias *et al.*, 2011, 2016). Still, this strategy entails reproductive costs, as up to 70% of all males born die during their first 3 years of life, and infant survival, particularly of male infants, is positively related to food availability (Dias *et al.*, 2015a, 2016). Therefore, besides its contribution to the understanding of sex allocation strategies, the present study has important conservation implications. As a consequence of habitat disturbance, females living in forest fragments produce less offspring of the sex that provides higher reproductive benefits,

and give birth to more infants of the sex that has lower survival probabilities. This scenario may be detrimental to population viability through its impact on population growth rates (Dias *et al.*, 2015b).

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