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RESEARCH ARTICLE

# A dog's life: Early life histories influence methylation of glucocorticoid (*NR3C1*) and oxytocin (*OXTR*) receptor genes, cortisol levels, and attachment styles

**Samantha L. Awalt**[1] | **Lidia Boghean**[2] | **David Klinkebiel**[2] | **Rosemary Strasser**[1]

[1]Neuroscience & Behavior Program, Department of Psychology, University of Nebraska Omaha, Omaha, Nebraska, USA

[2]Department of Biochemistry and Molecular Biology, Fred and Pamela Buffett Cancer Center, University of Nebraska Medical Center, Omaha, Nebraska, USA

**Correspondence**
Rosemary Strasser, Neuroscience & Behavior Program, Department of Psychology, University of Nebraska Omaha, 6001 Dodge St., Omaha, NE 68182, USA. Email: rstrasser@unomaha.edu

**Present address**
Lidia Boghean, Cancer Research Program, University of Nebraska Medical Center, Omaha, Nebraska, USA.

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## Abstract

Early life deprivation and stress can contribute to life-long, problematic consequences, including epigenetic variations related to behavior and health. Domestic dogs share human environments and social–cognitive traits, making them a promising comparative model to examine developmental plasticity. We examined 47 owner–dog dyads, including dogs rescued from abusive or neglectful environments, and matched control dogs for changes in DNA methylation of glucocorticoid (*NR3C1*) and oxytocin (*OXTR*) receptor genes previously shown to be affected by early life stress in other species including humans. We used an attachment paradigm, which included a separation event to examine cortisol levels and owner–dog attachment styles. Overall, dogs with adverse histories had different *NR3C1* methylation patterns as a function of age and less *OXTR* methylation than comparison dogs. Dogs with adverse histories did not differ in their cortisol change from baseline to poststressor from comparison dogs, but the change in cortisol was associated with *NR3C1* methylation. In addition, dogs with a history of early life stress had more insecure attachment styles; for every unit increase of *OXTR* methylation, the odds increased for insecure attachment style. This study demonstrates that adverse life histories lead to methylation differences, resulting in the hypothalamic–pituitary–adrenal (HPA) axis's dysregulation and differences in behavioral phenotypes.

**KEYWORDS**
attachment, cortisol, dog, early life stress, epigenetics, methylation, strange situation procedure

## 1 | INTRODUCTION

Early life stress (ELS) and deprivation can contribute to many life-long, problematic consequences, including epigenetic variation, stress responsivity, and behavioral effects. During prenatal and neonatal development, an individual's biology is organized and programmed to prepare for their environment (Mitchell et al., 2016; Van den Bergh et al., 2015). While embryonic development begins and is informed by its inherited DNA sequence, the DNA sequences are informed by internal and external factors, which affect DNA expression (He et al., 2011). This bidirectional relationship between biological predetermined factors and environmental influences is the foundation of epigenetics.

During early postnatal development, there are multiple time windows when the developing system is susceptible to environmental cues, known as experience-dependent events (Burns et al., 2018). This

amount of plasticity allows for environmental adaptivity. One adaptive response is the dynamic process of DNA methylation, which can occur early in life (Burns et al., 2018). Gene methylation is a process that impacts the transcriptional activity of DNA without altering the actual DNA nucleotide sequence by the addition of a methyl group to the cytosines of CpG dinucleotides near the gene promoter (Gouin et al., 2017; Razin, 1998). Methylation typically suppresses gene expression and is thought to be the most stable epigenetic modification (Hochberg et al., 2011).

### 1.1 | Hypothalamic–pituitary–adrenal axis, cortisol, and the glucocorticoid receptor gene (*NR3C1*)

Methylation of the glucocorticoid receptor (GR) gene (*NR3C1*) is one of the most heavily investigated genes associated with ELS (Palma-Gudiel et al., 2015). GR regulates the hypothalamic–pituitary–adrenal (HPA) axis, commonly known as the stress response system (Herman et al., 2016). The HPA axis connects the central nervous system with the endocrine system (Kudielka & Kirschbaum, 2005) via the release of glucocorticoids such as cortisol from the adrenal cortex (McEwen, 2000). As cortisol increases in circulation, it binds to GRs in the brain, which activate the negative feedback system and downregulate the HPA axis (McEwen, 2000). ELS produces long-term changes in HPA-axis activity (Levine, 1957), such as increased DNA methylation of the GR gene *NR3C1* (Palma-Gudiel et al., 2015; Weaver et al., 2004), which decreases the expression of GRs, which increases the amount of circulating cortisol during a stress event (Bird, 2002; Gray et al., 2017). Further, ELS and *NR3C1* methylation are related to an increased likelihood of major depressive disorder (Holmes et al., 2019), adverse social and emotional development in children (Folger et al., 2019), childhood behavioral problems (Gardini et al., 2022), anxious–depressive disorder, and decreased hippocampal connectivity (Palma-Gudiel et al., 2018). Additionally, given the regulating effects of GRs on the HPA axis, increased methylation of *NR3C1* leads to blunted cortisol reactivity for those with major depression (Bakusic et al., 2020).

While DNA methylation is commonly understood as one of the most stable epigenetic markers, there is supporting evidence that *NR3C1* methylation may not be as stable as previously understood. Growing evidence shows that age may play a role in understanding the relationship between early life histories and methylation. While many studies are published using infants/cord blood (Watkeys et al., 2018) or children (Holmes et al., 2019; Romens et al., 2015), some studies are beginning to explore the age spectrum. While it is more common to observe adulthood psychopathology with hypermethylation (Palma-Gudiel et al., 2015), there is evidence to the contrary. Women with chronic fatigue syndrome displayed *hypo*methylation (the loss of methyl groups from the DNA sequence) from peripheral blood samples compared to controls (Vangeel et al., 2018). Further, a rodent study found that adult females undergo *hypo*methylation with age, having higher methylation levels during infancy (Agba et al., 2017). This might suggest a beneficial time-sensitive period for methylation for typical individuals, followed by hypomethylation over time.

Deviations from typical methylation patterns can lead to HPA-axis dysregulation (Vangeel et al., 2018). In adolescents, higher *NR3C1* methylation levels are associated with flattened cortisol recovery slopes, indicating a delayed recovery time (Oberlander et al., 2008) and blunted cortisol response to social stress (Bunea et al., 2017). In a meta-analysis, ELS was associated with blunted baseline cortisol levels in blood samples, providing further evidence of the connection between ELS and HPA-axis dysregulation in children (Fogelman & Canli, 2018). Additionally, children who experienced harsh parenting styles at 2.5 years old have higher *NR3C1* methylation at 8.5 years old compared to children who did not experience harsh parenting styles (Lewis et al., 2020). Individuals who suffered childhood trauma had "dosage-dependent" cortisol responses to stress, such that those who experienced severe childhood trauma had higher cortisol peaks, and those with mild or unexposed trauma had moderate cortisol reactivity (Alexander et al., 2018). These results suggest a complex relationship between ELS, NR3C1 methylation, and their effects on the HPA axis, the consequences of which are often observed long after the ELS occurred.

### 1.2 | HPA axis, oxytocin, and attachment

Oxytocin is a peptide that functions as a hormone and neurotransmitter and interacts with the HPA axis (Rodrigues et al., 2009). Oxytocin is commonly termed the "prosocial neuropeptide" due to its facilitative role in social affiliative behavior (Ziegler et al., 2015) and plays a vital role in pair-bonding (Cavanaugh et al., 2016; Walum et al., 2012), social relationships (Feldman, 2012), mother–infant attachment (Nagasawa et al., 2012), and reproduction (Veening et al., 2015).

Oxytocin plays a crucial role in the attachment bond between individuals and, in some cases, between species. For example, it has been demonstrated in several studies that dogs will often form strong infant-like attachments to their owners (Konok et al., 2015; Topál et al., 1998; Udell & Brubaker, 2016), and humans release oxytocin during eye-gazing behavior with dogs, which subsequently increases oxytocin in the dogs (Nagasawa et al., 2015). The strange situation procedure (SSP), designed initially to assess infant attachment to a caregiver, was adapted for dogs (Ainsworth et al., 1978). Using this procedure, when the parent/owner leaves the room, secure children and dogs remain oriented to the door and greet the parent/owner during the reunion phase (Prato-Previde et al., 2003; Ryan et al., 2019). Dogs exhibit attachment styles to their owners as children do to their caregivers. They use their owners as a secure base in a novel environment to increase locomotive exploration and play behavior (Buttner et al., 2023; Palmer & Custance, 2008).

Reduced methylation of the oxytocin receptor (OXTR) gene is associated with reduced attachment anxiety in young adults (Ebner et al., 2019) and an increase in secure attachment styles (Haas et al., 2016). In comparison, maltreated children with atypical orbital frontal cortex display insecure attachment styles associated with increased OXTR methylation (Fujisawa et al., 2019). Similarly, border collies with OXTR methylation have increased hiding/fearful behavior compared to dogs

with less or no methylation (Cimarelli et al., 2017). When researchers examined OXTR polymorphisms in dogs and their owners to understand attachment behavior, Kovács and colleagues (2018) found that genetic variations in dogs and owners influence dog–owner attachment interactively. Additionally, in human studies, increased methylation of OXTR and *NR3C1* is associated with increased attachment avoidance (Ein-Dor et al., 2018), characterized by self-reliance and avoiding proximity to others (Ein-Dor & Hirschberger, 2016). Methylation of *NR3C1* is associated with an anxiety phenotype (Schmidt et al., 2019) and anxious attachment styles in children. Adults with fearful attachment styles have lower cortisol after a stressor compared to other attachment styles (Kidd et al., 2011, 2013), and insecure/anxious attachment styles are associated with an increase in cortisol reactivity when stressed compared to secure attachment styles (Smyth et al., 2015). In dogs, those classified as having a secure attachment style to their owners have been shown to have less cortisol reactivity during a threatening situation and when the owner is absent compared to insecurely attached dogs (Schöberl et al., 2016). Thus, OXTR and NR3C1 methylation and their effects on the HPA axis likely play an important role in attachment both in human and human–dog dyads.

### 1.3 | Dogs as a comparative model for developmental plasticity

Domestic dogs are an intriguing comparative model because of their human-like social cognitive abilities (Buttner, 2016; Hare & Tomasello, 2005) and the expression of various diseases, including psychological disorders such as canine compulsive disorder (Dodman et al., 2016; Tang et al., 2014) and anxiety disorders (Kurachi et al., 2017). Previous studies have found that dogs from adverse environments or early life deprivation (such as those confiscated due to abuse, animal hoarding situations, pet stores, or poorly run large-scale breeding operations often referred to as "puppy mills") experience more psychological disorders and behavioral issues, such as aggression, greater fear of social and nonsocial stimuli, and separation-related issues (insecure attachment) (Buttner & Strasser, 2022; McMillan et al., 2013; Pierantoni et al., 2011). Adverse early life histories in dogs have also been associated with higher cortisol levels and altered social behaviors when in the same novel stressful environment (Buttner & Strasser, 2022). Because dogs share the same ecological niche as humans and have been selected through domestication to share similar social–cognitive traits, they often mirror their owners (Kikusui et al., 2019). For example, previous research has shown both behavioral (Duranton & Gaunet, 2015, 2018; Duranton et al., 2017; Wanser et al., 2021) and hormonal synchronization with their owners (Buttner et al., 2015; Ryan et al., 2019; Sundman et al., 2019). The shared environment between dogs and humans makes them an attractive model for studying epigenetics and gene–environment interactions. The sequencing of the dog genome has led to several recent studies examining the genetic basis for behavioral traits (Morrill et al., 2022; Wayne & Ostrander, 2007) and human disease (Parker, et al., 2010; Shearin & Ostrander, 2010; Zapata et al., 2020). In this study, we investigated the effects of ELS on *OXTR* and *NR3C1* methylation, cortisol levels following a stressor, and attachment styles using dogs as model species.

### 1.4 | Current study

For the current study, we recruited owners of dogs that were rescued from adverse or deprived environments (e.g., abuse cases, hoarding situations, puppy mills, etc.) from shelter or rescue groups for the ELS group and a match control group of dogs without a history of neglect. These dogs meet the criteria of ELS with individuals showing varying degrees of low body weight (suggesting malnutrition), high housing density, early mother–pup separation, or other trauma. We investigated the relationship between the differential methylation status of the GR (*NR3C1*) gene and the oxytocin receptor (*OXTR*) gene with the presence or absence of ELS in dogs. An additional aim of this study was to determine how methylation status affects the HPA axis, which may subsequently affect health and behavior outcomes, including attachment styles to humans using the SSP. If ELS modifies DNA through epigenetic mechanisms, it was predicted that dogs from adverse conditions would have differential levels of DNA methylation on their GR (*NR3C1*) gene and the oxytocin receptor (*OXTR*) gene. If DNA methylation is associated with gene silencing, it was predicted that dogs from adverse backgrounds would be expected to exhibit greater cortisol change due to separation from their owner during behavioral testing.

## 2 | METHODS

### 2.1 | Participants and subjects

For this study, 47 dogs were recruited through fliers and emails sent to various shelters and rescue organizations in the area as well as social media, requesting owners of dogs that have come from known adverse conditions (rescued from puppy mills, hoarding, or abuse cases, etc.) or dogs that were known to have been raised in home environments by reputable breeders. Exclusion criteria included unknown early life history, endocrine regulating medications, and owners who have not lived with the dog for at least 3 months. Our dog sample consisted of 11 males and 13 females in the ELS group ($M_{age} = 4.67$, $SD = 3.23$) and 12 males and 11 females in the non-ELS group ($M_{age} = 4.61$, $SD = 3.57$). Our sample also consisted of 13 purebred and 15 mixes in the ELG group and 15 pure bred and 8 mixes in the non-ELS group. Dogs were categorized as either having no early life stress (non-ELS) or early life stress (ELS) histories after the owners filled out a questionnaire that asked open-ended questions such as: describe what you know about your dog's early life experience; the age that your dog was separated from its mother; and does your dog experience any sensitivities to stimuli, such as loud noises, different textures, or light. In general, dogs that were reported as having experienced early life deprivation had multiple accounts of early life negative experiences, such as malnourishment, mange, separation anxiety, and fearfulness, whereas owners reported no adverse experiences for dogs obtained from non-ELS con-

**TABLE 1** Descriptive variables based on early life background.

| Variable | Non-ELS ($M \pm SE$) | ELS ($M \pm SE$) | Statistical test |
|---|---|---|---|
| Age | 4.61 ± 0.75 | 4.67 ± 0.67 | $t(45) = -0.06, p = .95$ |
| Weight | 52.17 ± 5.16 | 43.16 ± 6.31 | $t(45) = 1.10, p = .28$ |
| Sensory sensitivities | 1.61 ± 0.21 | 3.50 ± 0.28 | $t(45) = -5.37, p < 001$*** |
| Shy/fearful behavior | 1.70 ± 0.25 | 3.17 ± 0.34 | $t(45) = -3.45, p = .001$** |
| Number of homes | 1.86 ± 0.23 | 1.23 ± 0.12 | $t(33) = 2.16, p = .07$ |
| Time in initial environment | 1.12 ± 0.24 | 0.61 ± 0.32 | $t(45) = 1.33, p = .18$ |
| Number of training classes | 2.39 ± 0.36 | 2.54 ± 0.38 | $t(45) = -0.20, p = .84$ |
| Frequency to a veterinarian | 3.33 ± 0.23 | 3.87 ± 0.22 | $t(45) = -1.25, p = .21$ |
| Reported illnesses | 1.71 ± 0.09 | 0.21 ± 0.19 | $t(45) = 4.93, p < .001$*** |
| Secure attachment | 13 | 5 | $\chi^2 = 4.95, p = .026$* |
| Insecure attachment | 7 | 17 | |

Abbreviation: ELS, early life stress.
*$p < .05$
**$p < .01$
***$p < .001$.

ditions (see Table 1 for additional information regarding demographics and descriptive variables).

All owners volunteered to participate and consented to their animals' genetic, behavioral, and endocrine analysis and the use of results for research purposes. All parts of this investigation were approved by the University of Nebraska Medical Center/University of Nebraska Omaha Institutional Review Board and Institutional Animal Care and Use Committee (IRB protocol # 412-17-EP; IACUC protocol # 17-024-06-EP).

## 2.2 | Procedure

Each dog–owner dyad followed the same subsequent procedural steps:

Step 1: owners brought their dogs to the Nebraska Humane Society for blood collection. Owners picked up their saliva packet at the humane society for Step 2.

Step 2: owners took an initial saliva sample before leaving their home for a baseline sample.

Step 3: the owner–dog dyad engaged in behavior testing at the University of Nebraska of Omaha (UNO).

Step 4: owners filled out a questionnaire at UNO.

Step 5: researchers collected the second saliva sample 20 min after owner–dog separation (see Section 2.2.1) at UNO.

### 2.2.1 | Behavior testing

Upon arriving at the University and before testing, dogs were given 20 min to acclimate to the environment to account for changes in cortisol levels due to travel or environment changes. Dogs and owners were then brought to the behavior testing room. The SSP was adapted to include only three 2-min episodes following an introductory episode and recorded on video (Thielke et al., 2017). The SSP was used to evaluate attachment styles between the owner–dog dyad, as used in other studies between mother and infant (Fonagy et al., 1991) and owner and dog (Topál et al., 1998). A novel, relatively empty room containing a chair for the owner at the front was used for behavior testing. Masking tape was used to construct lines on the floor to designate proximity to the chair, with one inner (approximately 1.52 × 1.52 m) and one outer (approximately 3 × 3 m) square, with toys placed in the outer square. The behavior test consisted of an introductory episode (30 s) where the dog was free to explore the room with the owner present. The owner was then asked to sit in a chair during Episode 1 (2 min) while the dog explores the room. At the end of the 2 min, the owner left the room for Episode 2 (2 min) before returning for Episode 3 (2 min), similar to what has been reported in other studies (Thielke et al., 2017).

Behavior coding was completed on a V-note Pro video coding software for Windows (version 3.3.0, https://v-note.org/). All three episodes of the SSP were video-scored for proximity maintenance to the owner by one of the researchers (L.B.) and later coded for other behaviors for another study. Due to dogs' time off camera (averages of time off camera: Episode 1: 10.63 s, Episode 2: 6.52 s, Episode 3: 6.89 s), behaviors were analyzed by the duration of behaviors as a proportion of time spent on camera. Pearson's correlations were used to check interrater reliability on a subset of behavior videos scored by another researcher. Scores for proximity to owner were found to be highly correlated for Episode 1 (inside box $r(3) = .996, p < .001$, outside box $r(3) = .998, p < .001$) and Episode 3 (inside box $r(3) = .935, p = .020$, outside box $r(3) = .924, p = .025$).

### 2.2.2 | Attachment style

During Episode 3, dogs were categorized as having a secure or insecure attachment style, dependent on their behavior upon reunion with

their owner. Dogs were categorized as having a secure attachment style if the dog met either of the following criteria: (a) greeted owner and within 30 s resumed exploration or play behavior, or (b) continued exploration or play behavior. Dogs were categorized as having an insecure attachment style if the dog met the following criteria: (a) greeted the owner and did not engage in explorative or play behavior within 30 s, or (b) remained in a fixed position.

### 2.2.3 | Blood and saliva sampling

Blood samples were collected because blood can predict tissue (amygdala and hippocampus) expression (Daskalakis & Yehuda, 2014; Na et al., 2014). Saliva was collected from dogs at two time periods for hormone analysis (saliva from owners was also collected at this time for another study). Saliva sampling is a practical, noninvasive method to assess hormone levels in dogs and humans (Dreschel & Granger, 2009; Lensen et al., 2015) highly correlated with plasma cortisol levels (Vincent & Michell, 1992). Owners received a saliva collection packet to obtain a baseline sample from their dogs and were sent a video demonstrating the procedure. A baseline sample was collected approximately 20 min before leaving home on the day of behavior testing. A postseparation sample was obtained 20 min after the separation stressor during the behavior test since cortisol reaches saliva after this time lag (for saliva sample execution with dogs, see Buttner, et al., 2023; Handlin et al., 2011; Lensen et al., 2015). Saliva samples were taken between 11:00 a.m. and 5:00 p.m. Given that cortisol levels peak after waking and gradually decline throughout the day, we recorded the time when the samples were collected, so the time of day could be used in future analyses. There was no effect of time of day on the subjects for any analyses.

Owners collected saliva from their dogs by holding a desirable treat in front of the dog's nose for approximately 30 s to stimulate salivation. Then, they were directed to place a piece of gauze in the dog's cheek pouches for approximately 2 min or until the gauze was fully saturated. Owners were then instructed to place their dog's saturated gauze strip into a centrifuge tube and store it in a freezer until leaving for the behavior testing session. Owners were asked to put the samples on ice for transportation to the testing site. Upon arriving at the behavior testing session, saliva samples were stored on ice, transferred to a laboratory freezer, and stored at −20°C until assay.

### 2.2.4 | Immunoassays

Saliva samples were warmed to room temperature upon assay. Dog samples were centrifuged at 5000 rpm for 5 min to extract saliva and separate it from debris. An enzyme immunoassay (EIA) was used to assess cortisol levels. Hormone assays were validated by creating displacement curves of halving dilutions from quality control saliva pools so that hormone standards were parallel in the 10%–90% binding range, and the difference in dilution resulted in an equivalent difference in the calculated concentration. Saliva samples were diluted to fall in this range. Microtiter plates were coated with CORT Ab (3.6.07) to quantify cortisol concentrations, diluted to 1:25,000 in bicarbonate coating buffer, and incubated for 12 h. CORT standards were diluted in phosphate-buffered saline (PBS), ranging from 1000 to 7.8 pg/well. Labeled CORT-HRP (R4866) was diluted 1:30,000 in PBS. After the 12-h incubation, 50 μL of PBS was added to each well, followed by 50 μL of the saliva samples or cortisol standards. After incubation, 50 μL of PBS was added to each well, followed by 50 μL of the saliva samples or cortisol standards. Then, 50 μL of HRP was added, and plates were incubated for 2 h. Free and bound hormones were separated, after which an EIA substrate (ABTS, $H_2O_2$) was added. A microplate reader was used to measure absorbance at 405 nm. Samples from the same individual were tested on the same plate in duplicate. The intra- and interassay coefficients of variation (CVs) were 6.7% and 10.3%, respectively.

### 2.2.5 | Dog cortisol change

Dog cortisol change was calculated by the percent change between the first and last cortisol collection, adjusting for the time between samples (Khoury et al., 2015). Time was adjusted by using the first saliva sample as a covariate.

### 2.2.6 | NR3C1

Two variants (1 and 2) of the canine NR3C1 gene were evaluated. The specific regions of each variant were selected based on previous human and rodent model studies that found correlations between methylation differences and ELS (Palma-Gudiel et al., 2015). Only one of the CpG sites investigated (*NR3C1* CpG 106) showed significant methylation differences based on ELS. *NR3C1* total methylation was calculated by the sum of the average percent methylation at CpG sites -106, -103, -99, -93, -82, and -42 relative to the transcription start site (see Figure 1).

### 2.2.7 | OXTR

The CpGs investigated for oxytocin receptor genes were selected based on a previous dog study, which found methylation differences at specific CpG sites in the OXTR promotor region (Cimarelli et al., 2017). The significant CpGs found relative to the transcription start site were -1383, -1371, -751, and -727, and thus primers were created to amplify the target regions that contained these CpG sites. Two primer sets were used to capture eight CpG sites. The first primer set captured CpG sites -1436, -1431, -1383, and -1371, while the second primer set captured CpG sites -751, -727, -687, and -660. Region 1 was defined as the CpG sites captured by the first primer set, which was further away from the OXTR transcription start site, and the percent methylation across CpG sites -1436, -1431, -1383, and -1371 was averaged and used for further analyses as methylation at Region 1. Region 2

| Chromosome Location | 38240862 | 38240859 | 38240856 | 38240852 | 38240846 | 38240835 | 38240830 | 38240824 | 38240814 | 38240807 | 38240805 | 38240795 | 38240789 | 38240783 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Relative to Transcription Start Site | -109 | -106 | -103 | -99 | -93 | -82 | -77 | -71 | -61 | -54 | -52 | -42 | -36 | -30 | Total Methylation |
| 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 37 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 16 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 23 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 41 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 38 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 25 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 6 | 0 | 0 | 0 | 0 | 0 | 0 |
| 19 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 18 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 39 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 40 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 28 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 31 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 42 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 46 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 26 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 17 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 47 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 35 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 43 | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 |
| 8 | 0 | 0 | 0 | 0 | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 |
| 45 | 0 | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 |
| 33 | 0 | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 |
| 22 | 0 | 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 6 |
| 24 | 0 | 4 | 0 | 0 | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 7 |
| 21 | 0 | 5 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 7 |
| 20 | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 | 0 | 0 | 8 |
| 36 | 0 | 9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 8 |
| 9 | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 | 0 | 0 | 8 |
| 4 | 0 | 0 | 0 | 0 | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 6 | 0 | 0 | 9 |
| 30 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 9 |
| 6 | 0 | 0 | 0 | 0 | 0 | 6 | 0 | 0 | 0 | 0 | 0 | 6 | 0 | 0 | 10 |
| 7 | 0 | 6 | 0 | 0 | 0 | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 |
| 3 | 0 | 0 | 0 | 0 | 0 | 6 | 0 | 0 | 0 | 0 | 0 | 7 | 0 | 0 | 11 |
| 34 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 11 |
| 32 | 0 | 5 | 0 | 0 | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 5 | 0 | 0 | 12 |
| 29 | 0 | 5 | 0 | 0 | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 6 | 0 | 0 | 13 |
| 10 | 0 | 4 | 0 | 0 | 0 | 6 | 0 | 0 | 0 | 0 | 0 | 6 | 0 | 0 | 13 |
| 13 | 0 | 4 | 0 | 0 | 0 | 6 | 0 | 0 | 0 | 0 | 0 | 6 | 0 | 0 | 13 |
| 14 | 0 | 4 | 4 | 0 | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 6 | 0 | 0 | 15 |
| 27 | 0 | 6 | 0 | 0 | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 7 | 0 | 0 | 15 |
| 15 | 0 | 4 | 0 | 4 | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 6 | 0 | 0 | 15 |
| 2 | 0 | 5 | 0 | 0 | 0 | 6 | 0 | 0 | 0 | 0 | 0 | 8 | 0 | 0 | 16 |
| 12 | 0 | 4 | 0 | 4 | 0 | 6 | 0 | 0 | 0 | 0 | 0 | 6 | 0 | 0 | 16 |
| 11 | 0 | 4 | 0 | 5 | 0 | 6 | 0 | 0 | 0 | 0 | 0 | 6 | 0 | 0 | 17 |

**FIGURE 1** *NR3C1* Variant 1. Blue-highlighted boxes represent non-ELS (early life stress) dogs, white boxes represent ELS dogs. Upon visual inspection, most non-ELS dogs have higher total methylation of all CpG sites compared to ELS dogs. Dogs with methylation for one CpG tend to have similar CpG methylation patterns. This indicates that methylation occurs in semipredictable patterns for non-ELS dogs. CpGs highlighted in yellow indicate CpGs that correspond to human CpGs that show differential methylation patterns in early life stress studies.

was defined as the CpG sites captured by the second primer set, closer to the OXTR transcription start site. The percent methylation across CpG sites -751, -727, -687, and -660 was averaged and used in further analyses as methylation at Region 2 (see Figure 2).

### 2.2.8 | DNA methylation procedure

DNA was extracted from whole blood samples using the Zymo Research: Quick-DNA Miniprep Plus Kit (D4068) following

(a)

| Chromosome Location chr20:..... (Broad CanFam3.1/canFam3) | 9358181 | 9358205 | 9358245 | 9358272 | |
|---|---|---|---|---|---|
| Relative to Transcription Start Site Isoform 1 | -751 | -727 | -687 | -660 | Average Methylation Region 2 |
| 45 | 30 | 12 | 0 | 0 | 10.5 |
| 14 | 22 | 11 | 13 | 0 | 11.5 |
| 38 | 20 | 10 | 10 | 7 | 11.75 |
| 27 | 20 | 10 | 10 | 8 | 12 |
| 7 | 20 | 11 | 10 | 8 | 12.25 |
| 13 | 20 | 11 | 10 | 8 | 12.25 |
| 33 | 17 | 10 | 11 | 11 | 12.25 |
| 36 | 21 | 9 | 11 | 8 | 12.25 |
| 40 | 27 | 11 | 11 | 0 | 12.25 |
| 4 | 21 | 9 | 10 | 10 | 12.5 |
| 43 | 20 | 10 | 11 | 10 | 12.75 |
| 16 | 22 | 11 | 11 | 8 | 13 |
| 6 | 22 | 10 | 12 | 9 | 13.25 |
| 2 | 24 | 11 | 12 | 7 | 13.5 |
| 10 | 23 | 10 | 11 | 10 | 13.5 |
| 24 | 23 | 11 | 12 | 9 | 13.75 |
| 25 | 27 | 9 | 12 | 7 | 13.75 |
| 31 | 21 | 11 | 12 | 11 | 13.75 |
| 5 | 26 | 11 | 11 | 8 | 14 |
| 9 | 25 | 11 | 11 | 10 | 14.25 |
| 11 | 27 | 11 | 11 | 8 | 14.25 |
| 12 | 24 | 11 | 11 | 11 | 14.25 |
| 18 | 24 | 11 | 11 | 11 | 14.25 |
| 21 | 22 | 11 | 12 | 12 | 14.25 |
| 23 | 27 | 10 | 11 | 9 | 14.25 |
| 26 | 24 | 11 | 10 | 12 | 14.25 |
| 28 | 23 | 12 | 11 | 11 | 14.25 |
| 29 | 24 | 11 | 12 | 11 | 14.5 |
| 34 | 25 | 13 | 11 | 9 | 14.5 |
| 1 | 24 | 12 | 13 | 10 | 14.75 |
| 8 | 20 | 12 | 12 | 15 | 14.75 |
| 15 | 27 | 11 | 11 | 10 | 14.75 |
| 17 | 26 | 11 | 10 | 12 | 14.75 |
| 20 | 26 | 11 | 11 | 11 | 14.75 |
| 30 | 25 | 11 | 11 | 12 | 14.75 |
| 47 | 21 | 12 | 14 | 12 | 14.75 |
| 22 | 27 | 12 | 11 | 10 | 15 |
| 3 | 26 | 11 | 12 | 12 | 15.25 |
| 19 | 25 | 12 | 13 | 12 | 15.5 |
| 39 | 28 | 10 | 13 | 12 | 15.75 |
| 41 | 27 | 11 | 13 | 12 | 15.75 |
| 35 | 27 | 14 | 12 | 11 | 16 |
| 37 | 30 | 11 | 13 | 10 | 16 |
| 42 | 28 | 12 | 14 | 11 | 16.25 |
| 32 | 27 | 13 | 16 | 10 | 16.5 |

(b)

| Chromosome Location chr20:..... (Broad CanFam3.1/canFam3) | 9358205 |
|---|---|
| Relative to Transcription Start Site Isoform 1 | -727 |
| 36 | 9 |
| 4 | 9 |
| 25 | 9 |
| 38 | 10 |
| 27 | 10 |
| 33 | 10 |
| 43 | 10 |
| 6 | 10 |
| 10 | 10 |
| 23 | 10 |
| 39 | 10 |
| 14 | 11 |
| 7 | 11 |
| 13 | 11 |
| 40 | 11 |
| 16 | 11 |
| 2 | 11 |
| 24 | 11 |
| 31 | 11 |
| 5 | 11 |
| 9 | 11 |
| 11 | 11 |
| 12 | 11 |
| 18 | 11 |
| 21 | 11 |
| 26 | 11 |
| 29 | 11 |
| 15 | 11 |
| 17 | 11 |
| 20 | 11 |
| 30 | 11 |
| 3 | 11 |
| 41 | 11 |
| 37 | 11 |
| 46 | 12 |
| 45 | 12 |
| 28 | 12 |
| 1 | 12 |
| 8 | 12 |
| 47 | 12 |
| 22 | 12 |
| 19 | 12 |
| 42 | 12 |
| 34 | 13 |
| 32 | 13 |
| 35 | 14 |

**FIGURE 2** *OXTR* variant. Heat map depicting methylation patterns of CpGs of interest. (A) Heat map depicting average percent methylation across Region 2 (containing CpG sites -751, -727, -687, and -660). Blue-highlighted boxes represent early life stress (ELS) dogs. Upon visual inspection, most ELS dogs have higher average methylation across Region 2 compared to non-ELS dogs. (B) Heat map depicting percent methylation at CpG site -727. Blue-highlighted boxes represent ELS dogs. Upon visual inspection, most ELS dogs have higher percent methylation at CpG site -727 than non-ELS dogs.

manufacturer-recommended procedures. Then, the DNA was treated with sodium bisulfite using the Zymo Research: EZ DNA Methylation Direct Kit (D5020) following manufacturer-recommended procedures.

To perform PCR reactions, 42 ng of bisulfite-modified DNA was used as a template. The PCR reactions were performed in a total volume of 50 μL for 35 cycles using Roche Diagnostic Corporation Fast-Start Taq DNA Polymerase (1.0 μM), MgCl2 solution (3.5 mM), dNTP (0.2 mM), sense primer (0.24 μM), and antisense primer (0.18 μM), with denaturation at 95°C for 30 s, annealing temperature for 45 s at optimized annealing temperature, and extension at 72°C for 1 min. Gel electrophoresis was performed on each sample to ensure the production of the proper PCR product. Lastly, according to the manufacturer's recommendations, the methylation percentage of each CpG was determined using a Qiagen Pyromark Q96 pyrosequencer.

Only one of the eight CpG sites investigated showed significant methylation differences based on early life background. Dogs that had experienced ELS had higher percent methylation at CpG -727 ($t(44) = -2.46$, $p = .02$). Methylation at Region 2, the region of DNA investigated that was closer to the *OXTR* transcription start site, was calculated by averaging percent methylation at CpG sites -751, -727, -687, and -660.

## 2.3 | Data analysis

### 2.3.1 | Initial analyses

Data were analyzed using RStudio v. 1.2.5001. Analyses used a significance threshold of $\alpha < .05$ (two-tailed). After removing samples from each time point that did not contain enough saliva for assays or were contaminated ($n = 7$), hormone levels were available for 36 dogs. Analyses included all available data points based on the specific analysis. Distributions were positively skewed (i.e., skewness index >3) for the following items and were normalized by taking a log transformation before statistical analysis: CpG-103, CpG-99, and CpG-77. CpG-103, CpG-99, and CpG-77 had zero variance and were excluded from further analysis. Baseline and postseparation cortisol levels were significantly correlated in dogs ($p < .05$), indicating stability in hormone levels. Dogs' baseline and postseparation cortisol levels were not associated with age or sex ($p > .05$).

Pearson's correlations were utilized to evaluate associations between hormone levels and other continuous variables (i.e., time of sample collection, weight, etc.). Additionally, *t*-tests were used to assess sex differences in hormone levels. Pearson's correlations were also used to assess individual CpGs with other continuous variables and the dog's background history. GR Variants 1 and 2 were assessed; Variant 2 showed no methylation for all subjects and was thus dropped from all further analyses.

The primary explanatory variables in each model were the dog's cortisol change, *NR3C1* total methylation, *OXTR* Region 2 total methylations, and dogs' proximity maintenance to their owner during the SSP. In addition, relationships among these were further explored in three-way interactions. Due to significant interaction terms by ELS, further analyses were done separately for each group for ease of interpretation. Parsimonious models were created through a backward selection process that retained variables where $p < -.05$.

# 3 | RESULTS

## 3.1 | DNA methylation, cortisol, and attachment styles

### 3.1.1 | Group differences

Overall, dogs with a history of ELS did not differ from the comparison dogs for total *NR3C1* methylation ($p > .05$). However, dogs with a history of ELS had less *OXTR* methylation across Region 2 compared to non-ELS dogs ($R^2 = .06$, $F(1, 44) = 4.05$, $p = .05$). Overall, dogs with a history of ELS did not differ in their cortisol change from baseline to poststressor (SSP) from the comparison dogs ($p > .05$).

### 3.1.2 | Cortisol change

To determine if *NR3C1* and *OXTR* methylation influenced the HPA-axis functioning, a regression was used to determine if the change in cortisol (baseline to poststress) was associated with methylation for all dogs. Cortisol change was associated with *NR3C1* methylation, where cortisol change decreased for each unit increase in *NR3C1* methylation ($R^2 = .12$, $F(1, 36) = 6.01$, $p = .02$); however, cortisol change did not differ based on the methylation of *OXTR* ($p > .05$), nor did the attachment styles of the dogs influence cortisol change ($p > .5$).

### 3.1.3 | Attachment

Methylation of OXTR mediates attachment style such that for every unit increase of *OXTR* methylation, the odds increased for insecure attachment style (odds ratio [OR] = 1.96, 95% confidence interval [CI]: 1.07, 4.08, $p = .05$). Further, when *OXTR* was removed from the equation, dogs from ELS groups were more likely to have insecure attachment styles (OR = 5.94, 95% CI: 1.61, 25.20, $p = .01$). There were no attachment style differences for *NR3C1* methylation ($p > .05$).

## 3.2 | DNA methylation, current age, and duration in early life environment

The three-way interaction for the length of time in early life environment, dog's age, and ELS group were significant for *NR3C1* methylation ($R^2 = .16$ $F(8, 26) = 1.89$, $p = .009$), after controlling for the number of homes (see Figure 3). Younger dogs with a history of ELS had more *NR3C1* methylation (*hyper* response), whereas older dogs had less *NR3C1* methylation (*hypo* response) ($t = -2.58$, $p = .02$) as the length of time in their initial environment increased. In other words, as ELS dogs

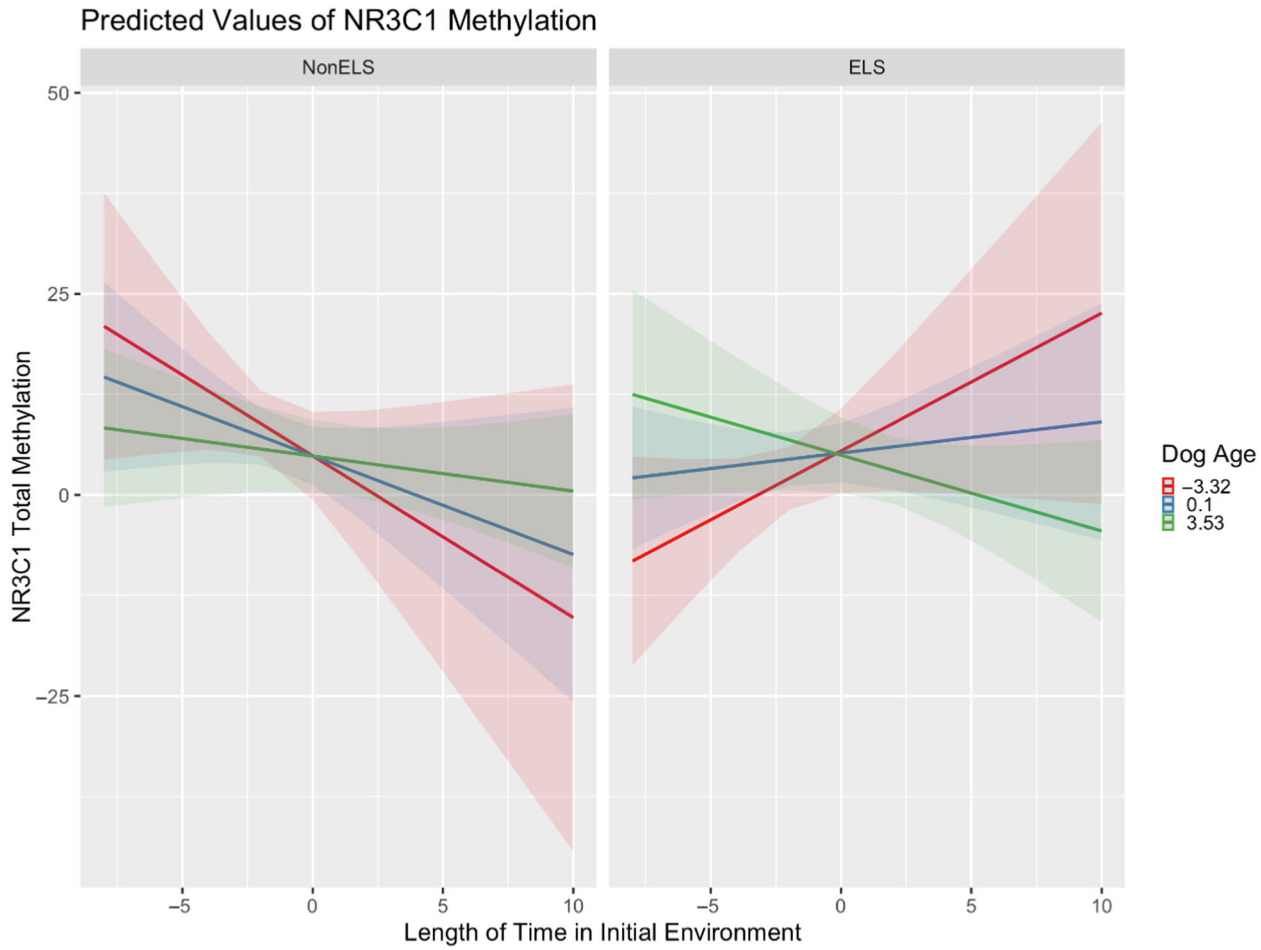


**FIGURE 3** Early life stress groups' *NR3C1* methylation for duration in early life environment and dog's age in a three-way interaction. The figure shows predicted values of NR3C1 for duration in early life environment and dog's age in a three-way interaction linear regression model. Shaded areas represent standard error. The dog's age is mean-centered, and lines represent 1 standard deviation above and below the mean (red = 1.4 years, blue = 4.7 years, green = 8.1 years). The length of time in the initial environment is mean-centered with predicted values ($M = 2.85$, $SD = 3.36$). This analysis used the number of homes the dog had before its current owner as a covariate. As the duration of stay in the early life environment increases, early life stress (ELS) dogs demonstrate an age effect, where younger dogs (red) have the highest level of methylation and older dogs (green) show hypomethylation, whereas non-ELS do not differ in age and show a downward slope of hypomethylation as the length of time in their initial environment increases.

spent more time in their initial environment, which would be aversive in the ELS group, it predicted a different pattern of *NR3C1* methylation in the younger ELS dogs more recently removed from that environment. Overall, dogs from non-ELS environments showed decreased NR3C1 methylation regardless of age.

### 3.3 | DNA methylation and cortisol changes in early life histories and attachment style

A three-way interaction was run to assess whether cortisol changes for dogs with a history of ELS were associated with NR3C1 and OXTR methylation. The three-way interaction for the ELS group, *NR3C1* methylation, and *OXTR* methylation was near significant for dog cortisol change ($R^2 = .15$ $F(7, 30) = 1.95$, $p = .09$) (see Figure 4). However, dogs with a history of ELS showed increased cortisol change when *NR3C1* was methylated compared to low or no *NR3C1* methylation ($t = -2.70$, $p = .01$).

We also ran a three-way interaction to assess whether cortisol changes occur for dogs with different attachment styles and whether those changes are associated with NR3C1 and OXTR methylation. The three-way interaction for attachment styles, *NR3C1* methylation, and *OXTR* methylation was significant for dog cortisol change ($R^2 = .58$ $F(8, 14) = 4.76$, $p = .05$) (see Figure 5). A two-way interaction for securely attached dogs was also significant; as *OXTR* methylation increased, dogs with *NR3C1* methylation had a steep decrease in dog cortisol change, compared to dogs with little or no *NR3C1* methylation, which showed a slight increase in cortisol change ($t = -4.12$, $p = .01$). A two-way interaction for insecurely attached dogs was also significant, such that for insecure attachments, as *OXTR* methylation increased, dogs with *NR3C1* methylation had a steeper increase in cortisol change compared to dogs with little or no *NR3C1* methyla-

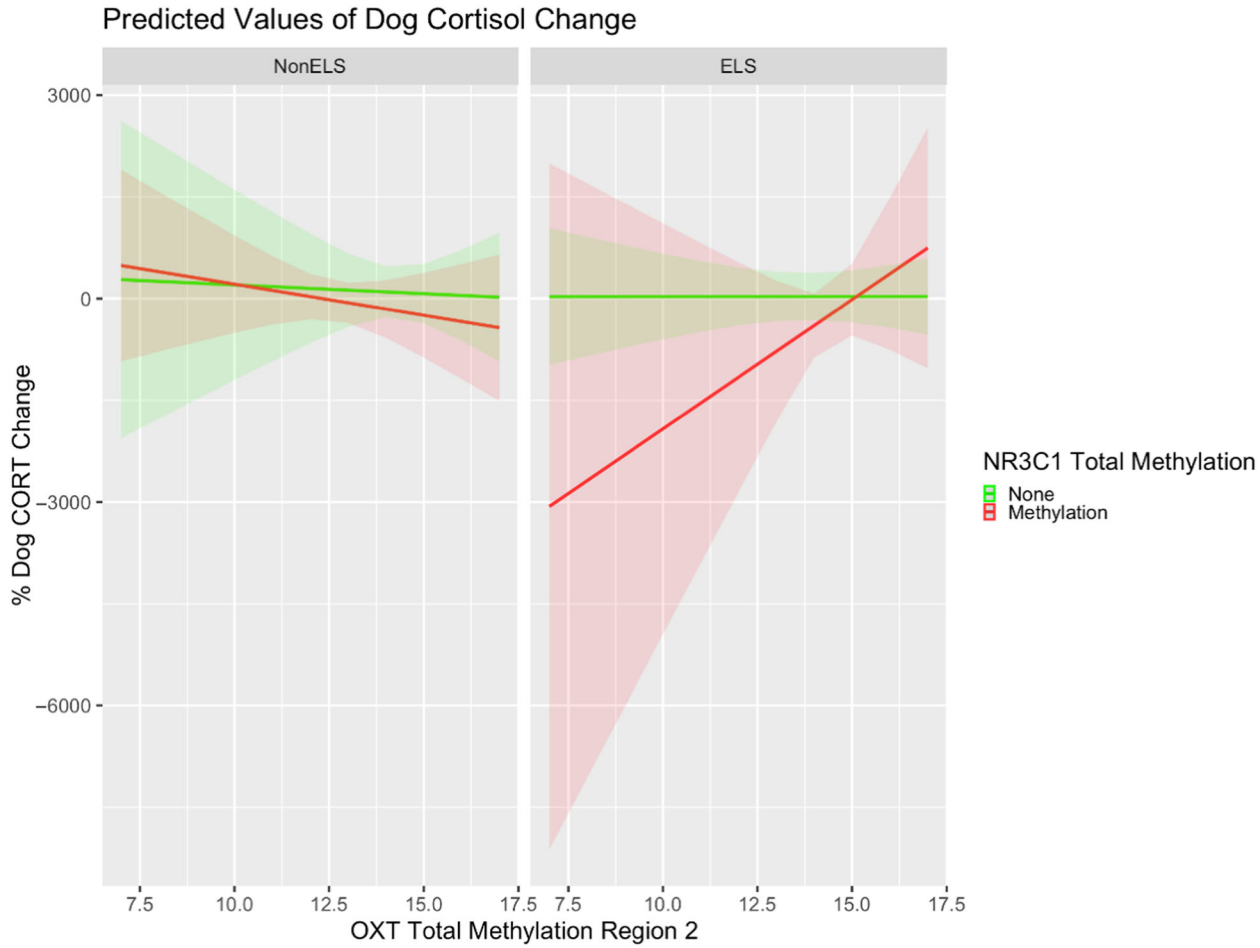


**FIGURE 4** Cortisol change for early life stress groups, *NR3C1* methylation, and *OXTR* methylation in a three-way interaction. The figure shows predicted values of dog cortisol change for early life stress groups, NR3C1 methylation, and OXTR methylation in a three-way interaction in a linear regression model. For visualization interpretation, NR3C1 total methylation is dichotomized and represents no methylation (none, $N = 20$) and methylation, which ranges from 4 to 17 (methylation, $N = 26$); standard error is represented by shaded areas. OXTR is mean-centered. Early life stress (ELS) dogs showed a trend for an increase in cortisol change from baseline to poststressor when NR3C1 methylation was present (red) compared to no NR3C1 methylation (green). Non-ELS dogs did not differ from each other based on NR3C1 methylation.

tion ($t = 3.81$, $p < .01$). This suggests that when methylation of both OXTR and NR3C1 genes is present, cortisol changes from baseline to poststressor are different depending on the attachment styles of the dogs.

### 3.4 | DNA methylation, early life histories, and attachment styles

Dogs with a history of ELS spent more time in proximity to their owner during the acclimation period (Episode 1) ($R^2 = .09$, $F(1, 44) = 4.28$, $p = .04$), as did all dogs characterized as those with insecure attachment styles ($R^2 = .24$, $F(1, 43) = 8.11$, $p < .01$). Further, a three-way interaction found that dogs with a history of ELS with higher *NR3C1* methylation spent more time in proximity to their owner during acclimation as *OXTR* methylation increased compared to non-ELS dogs ($R^2 = .36$, $F(8, 36) = 4.07$, $p < .01$), controlling for the dog's level of shy/fearful behavior upon acquisition (Figure 6).

During reunion with their owner (Episode 3), dogs with secure attachment styles spent more time away from their owners (less proximity maintenance) compared to insecure dogs ($R^2 = .29$, $F(1, 39) = 16.98$, $p < .01$). A three-way interaction found that dogs with ELS backgrounds with more *NR3C1* methylation spent more time in proximity to their owners during the reunion phase (Episode 3) as their *OXTR* methylation increased ($R^2 = .35$, $F(9, 27) = 2.59$, $p = .04$), after controlling for the age in which the dog left its mother as a puppy (Figure 7). Taken together, dogs from the ELS group that showed increased NR3C1 and OXTR methylation stayed close to their owner during the acclimation phase and after a reunion with them. No differences in separation phase (Episode 2) and methylation were observed.

## 4 | DISCUSSION

This study aimed to determine if methylation patterns of *NR3C1* and *OXTR* were associated with cortisol changes during the SSP and attach-

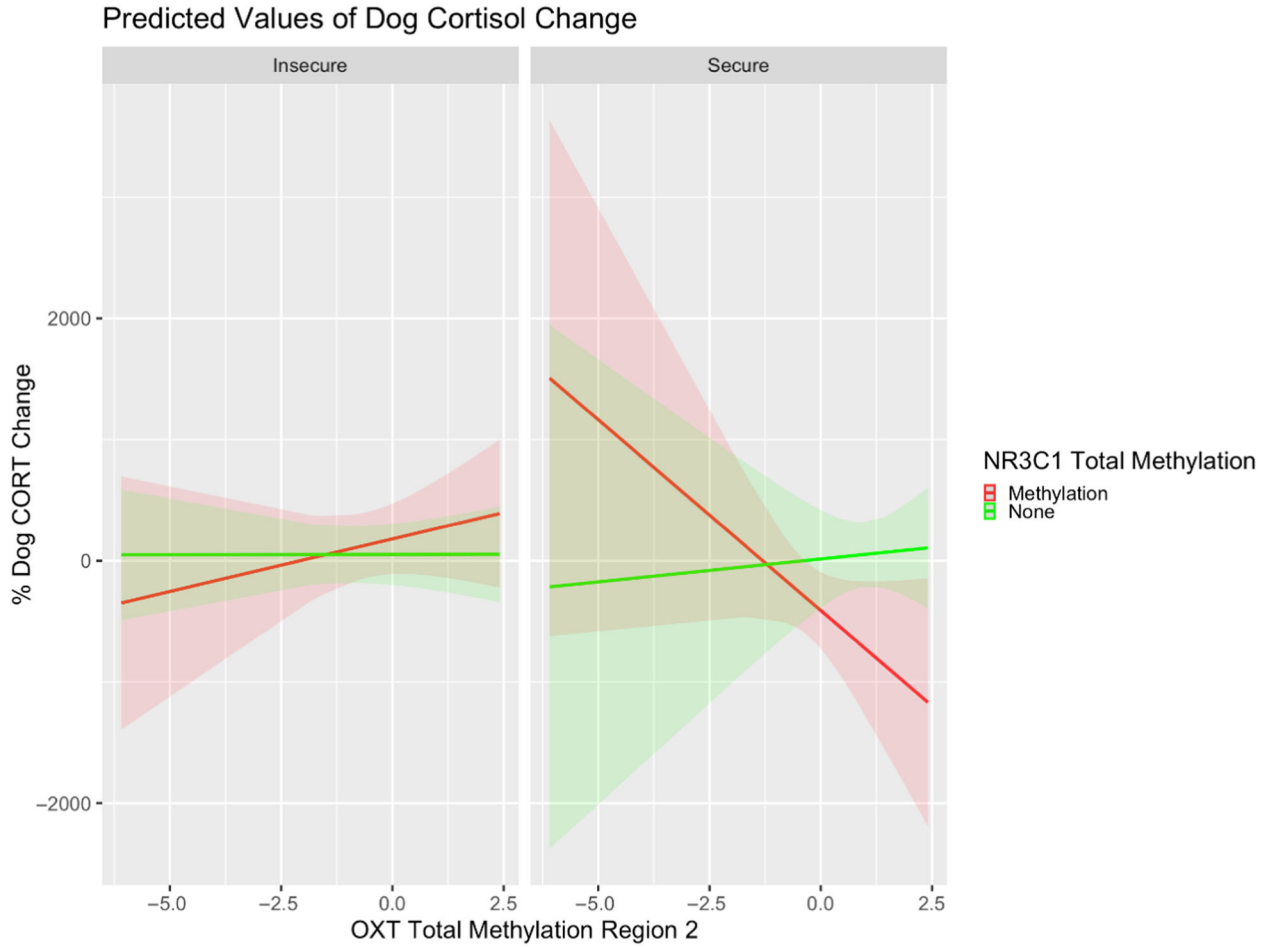


**FIGURE 5** Cortisol change for attachment styles, *NR3C1* methylation, and *OXTR* methylation in a three-way interaction. The figure shows predicted values of dog cortisol change for attachment style, NR3C1 methylation, and OXTR methylation in a three-way interaction in a linear regression model. For visualization interpretation, NR3C1 total methylation is dichotomized and represents no methylation (none, $N = 20$) and methylation, which ranges from 4 to 17 (methylation, $N = 26$); standard error is represented by shaded areas. OXTR is mean-centered. For secure attachments, as OXTR methylation increased, dogs with NR3C1 methylation (red) had a steep decrease in dog cortisol change, compared to dogs with little or no NR3C1 methylation (green), which showed a slight increase in cortisol change. For insecure attachments, as OXTR methylation increased, dogs with NR3C1 methylation had a steeper cortisol change increase than dogs with little or no NR3C1 methylation.

ment behavior in dogs with two different life histories. We predicted that changes in methylation patterns would exist and dogs with a history of ELS would have a blunted HPA axis and show insecure attachment behaviors. We found dogs with a history of ELS showed decreases in OXTR methylation and an increase in insecure attachment styles. Specifically, we found a mediation of *OXTR* methylation on attachment style such that as *OXTR* methylation increased, the odds increased for insecure attachment styles independent of ELS groups. This finding is consistent with previous studies showing that dogs (Kovács et al., 2018) and children (Chen et al., 2011) are more likely to develop insecure attachment styles when *OXTR* methylation increases. This is likely due to the reduced transcription of the oxytocin receptors. Thus, less oxytocin that binds to receptors may reduce the positive emotions associated with secure attachments (Maud et al., 2018). However, we found no support for *NR3C1* methylation mediating attachment styles.

We also examine cortisol change from baseline to poststressor in our dogs with different life histories. While some studies have found salivary cortisol levels were increased in dogs with insecure attachment styles (Riggio et al., 2022), dogs in our study with ELS did not differ in their cortisol change from baseline to poststressor, even though insecure attachment styles were associated with this group. While our findings regarding cortisol and ELS are less conclusive, cortisol change was associated with age and *NR3C1* methylation. First, we found an age-dependent relationship with time sensitivity between the dog's age and the duration of time they spent in the adverse environment. Specifically, younger dogs that spent more time in the adverse environment demonstrated *hyper*methylation, whereas older dogs that spent longer durations in the adverse environment displayed a *hypo*methylation for *NR3C1*. Previous research has demonstrated a link between genome-wide DNA *hypo*methylation in vertebrate organs during aging (Wilson & Jones, 1983), which may account for the


**FIGURE 6** Early life stress groups' proximity to the owner during the acclimation phase (Episode 1) of the Strange Situation Procedure (SSP) for *NR3C1* CpG 106 and *OXTR* methylation in a three-way interaction. The figure shows predicted values of early life stress groups' proximity maintenance to the owner during the acclimation phase (Episode 1) of the Strange Situation Procedure for NR3C1 CpG 106 and OXTR methylation in linear regression. For visualization interpretation, NR3C1 CpG 106 methylation is dichotomized and represents no methylation (none, $N = 26$) and methylation, which ranges from 3 to 8 (methylation, $N = 20$); shaded areas represent standard error. OXTR is mean-centered. Dogs with a history of early life stress (ELS) with higher NR3C1 methylation (red) spent more time close to their owner as OXTR methylation increased compared to non-ELS dogs after adjusting for the dog's level of shy/fearful behavior upon acquisition.

*hypo*methylation observed for older dogs but not younger ELS dogs. This age effect was similarly observed in adolescent children, where bad parenting style was associated with *hyper*methylation before 13.23 years old but switched to *hypo*methylation afterward for the *NR3C1* $1_F$ region (Van Assche et al., 2017). Additionally, longer durations in stressful environments can have a dosage effect on methylation status, where the higher number of traumatic events was associated with increased *NR3C1* methylation (Perroud et al., 2014; van der Knaap et al., 2014).

Next, we found that ELS dogs with methylation of the NR3C1 genes had an increased change in cortisol from baseline to poststressor, suggesting an elevated response. Childhood trauma shows a similar pattern, where increased *NR3C1* $1_F$ region methylation is associated with higher cortisol peaks poststressor (Alexander et al., 2018). Although previous studies have reported individuals with ELS backgrounds having higher *NR3C1* and *OXTR* methylation and an increase in cortisol change poststressor (Palma-Gudiel et al., 2015), our three-way interaction of ELS background, methylation of *OXTR* and *NR3C1* genes, and cortisol change did not reach significance. However, when we examined attachment styles regardless of background, we found securely attached dogs with increased *NR3C1* and *OXTR* methylation show a steep *decrease* in cortisol change. The opposite pattern emerges for insecurely attached dogs with increased *NR3C1* and *OXTR* methylation, showing cortisol levels increasing from baseline to poststressor. In sum, attachment style, *NR3C1* methylation, and *OXTR* methylation explained 58% of cortisol change. It is interesting to note that attachment styles could predict changes in cortisol during a stressor when the methylation status of both genes is similar. From a comparative perspective, it is also noteworthy that other human studies have demonstrated that increased *OXTR* methylation was associated with increased anxiety attachment (Ebner et al., 2019) and insecure attachment styles (Haas et al., 2016), and that the increase in both *NR3C1* and *OXTR* methylation was associated with insecure attachment styles (Ein-Dor et al., 2018). This is likely partly due to NR3C1 methylation

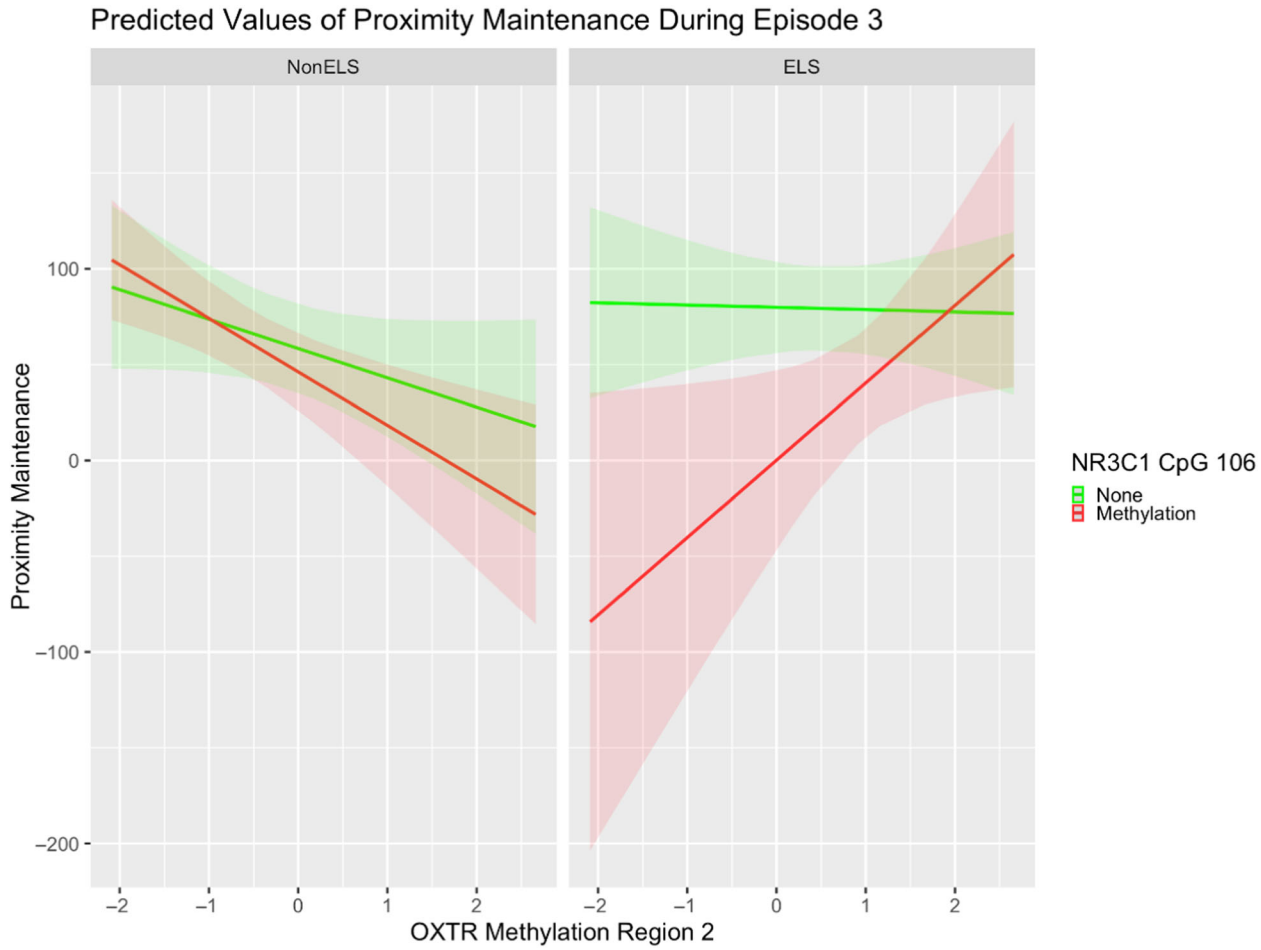


**FIGURE 7** Early life stress groups' proximity to the owner during the reunion phase (Episode 3) for *NR3C1* CpG 106 and *OXTR* methylation in a three-way interaction. The figure shows predicted values of early life stress groups' proximity to their owner during the reunion phase (Episode 3) for NR3C1 CpG 106 and OXTR methylation in linear regression. For visualization interpretation, NR3C1 CpG 106 methylation is dichotomized and represents no methylation (none, $N = 26$) and methylation, which ranges from 3 to 8 (methylation, $N = 20$); shaded areas represent standard error. OXTR is mean-centered. Dogs with a history of early life stress with more NR3C1 methylation (red) spent more time close to their owners during Episode 3 as their OXTR methylation increased ($R^2 = .35$, $F(9, 27) = 2.59$, $p = .04$), after adjusting for the age in which the dog left its mother as a puppy.

being associated with anxiety-type phenotypes (Schmidt et al., 2019), which would characterize an insecure attachment style.

This study also found differences in attachment behavior for dogs based on their early life histories. Dogs with a history of ELS and all dogs with insecure attachment styles would spend more time close to their owners than comparison dogs during the acclimation phase of testing. When considering methylation status, dogs with a history of ELS with higher *NR3C1* methylation spent more time close to their owner as *OXTR* methylation increased compared to non-ELS dogs, explaining 36% of the variation in proximity maintenance. In addition, dogs with a history of ELS with more *NR3C1* methylation spent more time close to their owners during the reunion episode as their *OXTR* methylation increased, explaining 35% of the variation in proximity maintenance. One potential interpretation for dogs maintaining proximity to their owner is that dogs use their owners as a social buffer to reduce their stress response (Buttner et al., 2023), as seen in various species (Hennessy et al., 2009). This interpretation would be consistent with previous research that demonstrated that dogs use their owners as a social buffer to attenuate their stress response during a stressor (Gácsi et al., 2013).

In contrast, if a dog was securely attached regardless of background, it spent less time in proximity to its owners during the reunion phase. We also found that non-ELS dogs spent less time with their owners regardless of *NR3C1* methylation. One interpretation of this finding is that securely attached dogs and non-ELS dogs used their owners as a secure base (Mariti et al., 2013; Palmer & Custance, 2008), meaning that after habituating to the room and when the owner was present during the reunion phase, the dogs felt comfortable exploring their environment similar to what is seen in children (Allen et al., 2003). The prevalence of insecure attachment styles in dogs with a history of ELS may influence how they respond to a stressful event with their owner present. Previous research with dogs with a history of ELS has also reported insecure attachment styles and hypothesized that these dogs use their owner to buffer stress (Buttner et al., 2023).

## 5 | CONCLUSION

This study's DNA methylation patterns, cortisol change, and insecure attachment styles in dogs were likely due to prolonged stress in impoverished conditions during critical periods of development. This is further supported by studies in dogs from poorly run large-scale breeding operations or hoarding situations that have less desirable behaviors as adults and worse mental and physical health scores than dogs from other sources (McMillan et al., 2011, 2016; McMillian et al., 2017; Stella et al., 2019; Wauthier & Williams, 2018). This study supports the ill effects of ELS into adulthood in domestic dogs as demonstrated in other social species (for review, see Dettmer & Chusdy, 2023). Animals and humans that have experienced ELS or adversity often display behavioral and physiological differences well into adulthood. Exploring these relationships across a range of species and those that live in the same ecological niche as humans, such as dogs, could also shed light on the mechanisms of developmental plasticity and inform us regarding the welfare and subsequent care of dogs raised with different life histories.


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### CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

### DATA AVAILABILITY STATEMENT

The data are publicly available.


### ORCID

*Rosemary Strasser* https://orcid.org/0000-0003-4983-3057


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