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RESEARCH ARTICLE

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A mechanism for population self-regulation: Social density suppresses GnRH expression and reduces reproductivity in voles

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Abstract

1. Nearly 100 years ago, Charles Elton described lemming and vole population cycles as ecological models for understanding population regulation in nature. Yet, the mechanisms driving these cycles are still not fully understood. These rodent populations can continue to cycle in the absence of predation and with food supplementation, and represent a major unsolved problem in population ecology.
2. It has been hypothesized that the social environment at high population density can drive selection for a low-reproduction phenotype, resulting in population self-regulation as an intrinsic mechanism driving the cycles. However, a physiological mechanism for this self-regulation has not been demonstrated. We manipulated population density in wild meadow voles *Microtus pennsylvanicus* using large-scale field enclosures over 3 years and examined reproductive performance and physiology.
3. Within the field enclosures, we assessed the proportion of breeding animals, mass at sexual maturation, and faecal androgen and oestrogen metabolites. We then collected brain tissue from juvenile voles born at high or low density, quantified mRNA expression of gonadotropin-releasing hormone (GnRH) and oestrogen receptor alpha (ERα) and measured DNA methylation at six CpG sites in a region that was highly conserved with the mouse GnRH promoter.
4. At high density, there was a lower proportion of reproductive animals. Juvenile voles born at high densities had reduced expression of GnRH in the hypothalamus, accompanied by marginally lower faecal sex hormone metabolites. Female juvenile voles born at high density also had higher methylation levels at two CpG sites while males did not, aligning with prior observations that females (but not males) from high-density environments retain reduced reproduction long term.
5. Our results support a physiological basis for population self-regulation in vole cycles, as altering population density alone induced reproductive downregulation at the hypothalamic level. Our results demonstrate that altering the early-life social environment can fundamentally impact reproductive function in the brain. This, in turn, can drive population demography changes in wild animals.

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KEYWORDS
androgens, early life programming, epigenetics, field study, methylation, population cycles, reproductive suppression, vole and lemming cycles

1 | INTRODUCTION

No population increases without limit. Population self-regulation (or intrinsic regulation) has been a major concept in ecology, but concrete evidence for its underlying mechanism(s) remain elusive. Intrinsic regulation is a special case of population regulation and density dependence (for more on the latter concepts, see Krebs, 2002; Sinclair, 1989). It refers to regulation by factors internal to the population such as social behaviour, dispersal and population genotypes and phenotypes (Krebs, 2002, 2009; Oli, 2019; Ostfeld et al. 1993; Wolff, 1997). This is in contrast to extrinsic (or environmental) factors such as food resources, predation, and disease, which can all be involved in other density-dependent population processes.

The concept of intrinsic regulation was largely formulated from observations on arvicoline rodent (vole and lemming) population demography (Chitty, 1960). These small mammals are key herbivores sustaining much of the food web throughout northern ecosystems. Over 3–5 year cycles, voles and lemmings will increase to extremely high densities (the 'peak phase') and then decline to low densities (the 'low phase'; Krebs et al., 1973). These population cycles were documented about a century ago (Elton, 1924), and are still the subject of intense research today (Johnsen et al., 2019; Oli, 2019; Sundell et al., 2019; Van Cann et al., 2019). The enigma of the cycles is that declines can occur even in the absence of predation, food limitation, or disease (Boonstra et al., 1998; Chitty, 1960; Krebs, 2013). Furthermore, experimental predator exclusion and food supplementation of cycling populations do not always prevent declines (Cole & Batzli, 1978; Marcstrom et al., 1988; Maron et al., 2010). Though these environmental factors may be sufficient for declines, they are not necessary. Hence, the declines must also be due to changes in the voles themselves, that is, intrinsic regulation. However, a mechanism for this self-regulation has never been demonstrated, and the causes of vole and lemming cycles remain an unsolved problem in population ecology.

Consistent with intrinsic regulation, voles at peak density have socially induced reproductive suppression and poorer survival such that the death rate exceeds the birth rate, precipitating the population crashes (Chitty, 1967). This change was hypothesized to be a result of density-dependent selection for a high aggression and low-reproduction morph at peak density, alternating with selection for a low aggression and high reproduction morph at low density (Chitty, 1967). The intraspecific competition was hypothesized to be for breeding territories, which is intensified at high densities. Female voles show exclusive spacing behaviour during the breeding season, and low female overlap is correlated with better offspring survival (Boonstra, 1977; Boonstra & Rodd, 1983; Madison, 1980; Mihok, 1979; Ostfeld et al., 1988). Numerous studies have also demonstrated qualitative differences in voles at different phases of the population cycle. At peak densities, voles have shorter breeding seasons and delayed sexual maturation (Boonstra, 1985; Krebs et al. 1969; Norrdahl & Korpimäki, 2002; Novikov et al., 2012). Hence, there is a lower proportion of reproductive adults in the population, which is maintained into the decline even as densities drop (Getz et al., 1979; Krebs et al., 1969; Mihok & Boonstra, 1992). The phase-dependent reduction of reproduction also appears to have a degree of permanence and heritability, and so is not solely an immediate response to conspecifics at high density (e.g. as in Massey & Vandenbergh, 1980). Voles captured from different population densities and brought into the laboratory retain reduced reproduction if they were captured from a peak/declining population relative to those captured from a low/increasing population (Bian et al., 2015; Mihok & Boonstra, 1992). This reproductive signature of the natal environment is more pronounced in females than in males, with males being more plastic and rebounding rapidly to a high reproduction phenotype (Bian et al., 2015; Mihok & Boonstra, 1992).

In the original 1967 formulation of Chitty's hypothesis, the trade-off in phenotype between a high- and low-reproduction morph was proposed to be the result of rapid selection on a naturally occurring genetic polymorphism (Chitty, 1967). This was rejected due to low paternal heritability of these traits in voles (Boonstra & Boag, 1987) and lemmings (Boonstra & Hochachka, 1997). Instead, we hypothesized that vole population density alters the expression of genes involved in reproductive activity, and that these expression differences are epigenetically programmed via changes in DNA methylation. Environmental conditions in early life (including both in utero and postnatal) can programme the epigenome to adaptively alter phenotype without changes in the underlying genetic code. This can allow for phenotypic plasticity in response to environmental conditions but also permanence in the programmed phenotype across life (Bossdorf et al., 2008; Verhoeven et al., 2016). Though there are various epigenetic mechanisms, DNA methylation has been the most well studied in ecology (Bossdorf et al. 2008; Verhoeven et al., 2016). DNA methylation is the attachment of a methyl group onto DNA, usually to the cytosine in CpG dinucleotides in the genome, and often represses gene transcription when it occurs in a gene promoter (Berger, 2007).

In the vole system, such changes could allow for alteration to the reproductive phenotype in response to density, which is stable across life, so that animals born in the peak would maintain a low-reproduction phenotype into the decline. To test this, we experimentally altered meadow vole *Microtus pennsylvanicus* population density by constructing large-scale, terrestrial predator-proof field enclosures. Enclosure populations were monitored by weekly live-trapping May through August (the typical breeding period for this species). This design was repeated in three summers (2016–2018), resulting in 11 low-density enclosures and 7 high-density enclosures.

In August of each year of the study, a subset of sexually immature juveniles was collected from the enclosures to assess early life differences in neural gene expression (mRNA) and cytosine methylation of candidate genes in the hypothalamic–pituitary–gonadal axis. In sexually immature juveniles from both the high- and low-density enclosures, we measured mRNA expression of two critical reproductive genes. The first gene, GnRH1, encodes gonadotropin-releasing hormone (GnRH), the reproduction 'master regulator' at the head of the hypothalamic–pituitary–gonadal axis (Maruska & Fernald, 2011). GnRH has been demonstrated to be socially modulated in vertebrate systems, and influenced by factors such as dominance status, mating calls (Maruska & Fernald, 2011) and photoperiod in seasonally breeding species, including voles (Kriegsfeld & Nelson, 1999). The second gene, ESR1, encodes oestrogen receptor alpha (ERα). ERα expression in the hypothalamus and medial amygdala has been associated with trade-offs in parental care and aggression (Horton et al., 2014), which could be key in the vole system. We predicted that high density should be associated with a decrease in GnRH expression, as high density has been found to suppress reproduction in voles. Furthermore, we predicted that high density should be associated with an increase in ERα expression in the medial amygdala, which has been previously associated with reduced prosocial behaviour in voles (though in males: Cushing et al., 2004; Stetzik et al., 2018) and a decrease in the hypothalamus, which has been associated with reduced maternal behaviour in voles and rats (Champagne et al., 2006; Feng et al., 2019).

2 | MATERIALS AND METHODS

2.1 | Field enclosures

Enclosures (Figure 1) were constructed in spring 2016 at the Koffler Scientific Reserve (King City, ON, Canada; 44°01′48″N, 79°31′56″W). To prevent animals from escaping and those from surrounding grasslands from entering, each enclosure was a fenced 25 × 25 m (0.0625 ha) area surrounded by metal hardware cloth (1/4 inch mesh) extending 0.6 m above and 0.6 m below ground and capped with aluminium (to prevent voles from climbing out). The outer perimeter was surrounded by additional Vexar plastic fencing to a height of 1.5 m and by an electric fence to deter terrestrial predators from entering the grids. We found no evidence of predation in the enclosures during any year of the study. Prior to the start of the experiment, we live-trapped to remove any existing small mammals in the enclosures (*Microtus pennsylvanicus*, *Peromyscus* sp.). No *Peromyscus* sp. were found in the enclosures during the experiment. Each year in the spring, prior to introducing animals, all enclosures were fertilized (21% nitrogen, 7% potassium and 7% potash) to support plant growth.

2.2 | Establishing enclosure populations

During the spring of each of the 3 years of the study (2016, 2017 and 2018), founding animals were live-trapped in the meadows around Koffler Scientific Reserve approximately 1 month prior to the start of the experiment (late March–early April of all years). At that time, most females were not yet reproductive. These voles were then individually housed in the University of Toronto Scarborough wildlife research facility in 91.5 × 61 × 46 cm polypropylene cages. Voles were provided with cotton nesting material, ad lib water, apple slices, oats, and rabbit chow (LabDiet; 14.5% protein, 22.6% crude fibre, 2.8% fat). They were maintained at a temperature of 15–20°C and a natural photoperiod. The purpose of this month-long holding period was to ensure that all founding females released into the enclosures were not pregnant (i.e. it covered the 21-day gestation period), and to ensure that all animals were released simultaneously. In early May of each year, all founding animals were released into their respective enclosures. Thus, all animals born within the field enclosures were conceived at the intended density treatment. Voles were introduced at starting densities of either 4–6 animals or 20–24 animals per enclosure, based on naturally occurring low and high population densities documented nearby (Boonstra, 1989; Boonstra & Rodd, 1983). The sex ratio of the founders was ~60% female and ~40% male, which was the naturally occurring sex ratio of these nearby populations. Low-density enclosures were periodically cropped, ensuring that high- and low-density treatments maintained disparate densities over the summer. Enclosures were trapped out each year at the end of the study (last week of August) to enumerate all animals were present and ensure that vegetation was not damaged over the winter by high-density populations in preparation for the next year. Hence, enclosure populations are not descended from populations present overwinter from the previous year. Treatment enclosures were switched each year so that high-density populations were in enclosures that had previously held low-density populations, to counteract the potential for cumulative vegetation damage. Owing

FIGURE 1 The eight field enclosures constructed at Koffler Scientific Reserve viewed from a Google Earth satellite image (left) and from the side (right). Each enclosure was 25 × 25 m, resulting in an outer perimeter of 50 × 100 m, and separated by metal hardware cloth

to insufficient voles being captured in some springs, not all enclosures were used every year. Any enclosures that were not able to be used in a given year were due to lack of sufficient animals trapped for the founding population. For example, in 2017, only ~50 animals were trapped around Koffler Reserve for the founding population. As a consequence, only 2 high-density and 2 low-density treatments were created that year. These yearly differences in availability of spring founding animals may be due to overwintering weather conditions (freezing rain), natural population fluctuations in the area, or other environmental factors (and for this reason year was always included as a random factor in the subsequent analyses).

2.3 | Live-trapping and population demography

All enclosures contained a 5 × 5 grid of Longworth live-traps spaced 5 m apart. High-density enclosures contained two traps at each grid point (50 traps), whereas low-density enclosures contained one trap at each grid point (25 traps). Low-density enclosures were maintained at low density by cropping them continuously throughout all summers by releasing animals outside of the fences. Animals released during cropping were juveniles of weaning weight, to reduce potential artefacts from removing the original founding animals, which may have social dominance effects on the population. During all years, voles were live-trapped on a weekly basis from early May through August (16 trap weeks a year). Live-traps were baited with oats and contained cotton nesting material, set at 04:00 hr and checked at 08:00 hr. When captured, voles were ear tagged with an identifying fingerling fish tag (Ameri-marks). Body mass was measured using Pesola spring scales (±1 g), the grid location was recorded, and reproductive condition was determined. Females were considered sexually mature if they had a perforate vagina, and breeding if they were lactating and had perforate vagina and/or opening of the pubic symphysis. Males were considered breeding if they had a scrotal testes position. For each trapping week, population density was calculated as minimum number alive per enclosure (MNA; Krebs, 1966). In field studies, MNA is often subject to tapering bias in the first and last trapping weeks; the first and last weeks are underestimated due to a lack of cumulative data before or after those points, respectively (Pocock et al., 2004). However, since enclosures were trapped out in the last week of the experiment (setting and checking traps until no more individuals were captured), this should reduce such biases in MNA calculations.

Treatment differences in average enclosure MNA across the study period were compared to check if any enclosures markedly deviated in density from the treatment they were intended to be. The summer average MNA of the high-density enclosures ranged from 18 to 36 (mean of the average densities = 28). The summer average MNA of the low-density enclosures ranged from 3 to 15 (mean of the average densities = 12). One enclosure in 2017 which was initially intended to be high density but failed to stay at high density (average MNA of 15 animals). This change appeared to be due to a depletion early on in the study (May–June) of founding animals. Missing animals were not recovered, and may have either escaped through a weakness in the fence (gap or tunnel) or died in the enclosure and were never found. We reassigned this enclosure to low density. Any animals that were found to change grids during the study (by tunnelling) were included in density estimates but were not included in analysis of treatment differences in demography or physiology, as their density history was varied. In all, 16 animals throughout the course of the entire study were documented as changing grids. All animal work procedures, including handling and collection, were approved by the University of Toronto animal care committee and done in accordance with University of Toronto animal use protocol #20011477.

2.4 | Analysis of faecal sex hormone metabolites

Faeces were collected from the tunnels of live-traps intermittently during the study. On the days of faecal sample collection, live-traps were set at 06:00 hr and checked at 08:00 hr to minimize the time the animals were confined in the traps. Since a single animal could enter a live-trap at a time, the identity of each sample was known. Urine-contaminated samples were not collected. The faecal samples were collected from tunnels with forceps and then stored temporarily in a cooler on ice packs until transfer to a freezer at University of Toronto Scarborough that day. Faecal samples were extracted using previously described procedures (Palme et al., 2013; for meadow voles specifically see Edwards et al., 2019). In brief, they were freeze-dried, weighed and extracted in 1 ml 80% methanol. Faecal androgen metabolites (FAMs) were measured using an epiandrosterone enzyme immunoassay that measures androgen metabolites with a 17-oxo configuration (Palme & Möstl, 1994). This assay was tested in both sexes, as changes in FAM levels have been associated with reproductive state differences in other female mammals (e.g. *Tamiasciurus hudsonicus*; Dantzer et al., 2011; *Eubalaena glacialis*; Rolland et al., 2005; *Rhinoceros unicornis*; Schwarzenberger et al., 2000). Oestrogen metabolites were measured in faecal samples from females using a total oestrogen immunoassay (Palme & Möstl, 1994). As a biological validation of these assays in meadow voles, levels in reproductive and non-reproductive animals were compared. Because only the FAM assay was able to differentiate between reproductive and non-reproductive animals, FAM data were used while faecal oestrogen metabolite data were not analysed further (see Appendix S1).

2.5 | Brain tissue collection

In late August of each year, brain samples were collected. Live-traps were set at 06:00 hr, and checked at 08:00 hr. Juvenile animals were collected with the criteria that they had not yet reached reproductive maturity (females had no vaginal perforation or lactational tissue and males had testes that had not descended into the scrotum) and were young born in the enclosure. These animals were likely born in from

mid-July to early August, though the exact ages were not known. Voles were transported in the live-traps to the Koffler Reserve laboratory (<5 min from the field enclosures). Proceeding in a random order, each animal was removed from the trap and rapidly euthanized via isoflurane overdose. All of this occurred within 3–4 hr of capture. The brain was removed and placed on sterile aluminium foil inside a cooler filled with dry ice. After freezing, brains were wrapped in parafilm and transported on dry ice to the University of Toronto Scarborough where they were stored at −80°C until analysis. The medial pre-optic area of the hypothalamus and the medial amygdala were targeted based on brain landmarks in the Allen Mouse Brain Atlas (Lein et al., 2007). These regions were dissected from 30 μm microsections made with a Cryostat (Leica CM3050S, Leica Biosystems). DNA and mRNA were extracted from the tissue from each region (MasterPure Complete DNA and RNA Purification Kit, Epicentre).

2.6 | mRNA quantification by RT-qPCR

mRNA was converted to cDNA (High Capacity cDNA Conversion Kit, Applied BioSystems) and quantity was assessed using a spectrophotometer (Nanodrop ND-2000C, Thermo Scientific). Primers for amplification were designed using NCBI primer-BLAST (Ye et al., 2012) based on congener *M. ochrogaster* sequences, NCBI GenBank assembly accession GCA_000317375.1 (Table S1). When testing primers, products were run on an agarose gel to determine that they were the expected size. The expression of two candidate genes and four reference genes was quantified using StepOne Plus real-time PCR software. qPCRs were performed using Fast SYBR Green master mix (Applied Biosystems) and 30 ng of cDNA. A standard curve was generated from serial dilutions of pooled cDNA to ensure that samples fell within a quantifiable range. Samples that fell below the standard curve or did not properly amplify (failed to show a single melt peak) were not used. Samples were assayed in triplicate. The reference genes glyceraldehyde phosphate dehydrogenase (GAPDH) and actin beta (ACTB) were selected using the R package NormqPCR (Perkins et al., 2012), and a normalization factor was calculated for each sample using their geometric mean (see Appendix S1 for more information). Different treatments, sexes and years were mixed across qPCR plates ($n = 3$ plates per gene) to minimize potential plate-driven effects on these variables. The coefficient of variation of a control sample run on all plates was <5% for all genes. Relative gene expression of candidate genes for each sample was determined using the delta CT method (Schmittgen & Livak, 2008), and the final values analysed were the −ΔCT normalized to the series mean of each candidate gene.

2.7 | CpG site methylation analysis by bisulphite pyrosequencing

To choose CpG sites that may regulate GnRH expression, CpG sites were chosen in the area of the *M. pennsylvanicus* genome that is highly conserved with the GnRH promoter region of mice and rats. As *M. pennsylvanicus* has no published reference genome, we searched for CpG sites up to 1.5 kilobases (kb) upstream of the transcription start site of GnRH in the *M. ochrogaster* genome using the UCSC genome browser (Kent et al., 2002). One of the more CG dense regions in this 1.5 kb range was around a sequence that is highly similar to the early growth response protein (Egr-1, also known as NGFI-A) transcription factor binding site in the mouse proximal GnRH promoter region (5′-GCGGGGAAG-3′; DiVall et al., 2007). In mouse GnRH cells, Egr-1 binding in the GnRH promoter mediates the insulin signalling that increases GnRH expression (DiVall et al., 2007). Furthermore, Egr-1 transcription factor binding sites in the glucocorticoid receptor gene (NR3C1) have been the target of methylation studies in rodents (Weaver et al., 2004) and in a single GnRH promoter methylation study in fish (Alvarado et al., 2015). Aside from this, to our knowledge, there is only one other species where DNA methylation changes in the putative GnRH promoter have been assessed, and this is in macaques which have a unique CpG island in the GnRH promoter that rodents do not have (Kurian & Terasawa, 2013). Thus, the region conserved with the mouse proximal Egr-1 binding site was chosen in voles.

To test whether these CpG sites were the conserved in *M. pennsylvanicus*, we amplified this region of the genome using primers based on the same region in *M. ochrogaster* (Figure S1). PCR products were sent to The Centre for Applied Genomics (SickKids Hospital, Toronto, ON, Canada) for Sanger sequencing. The results showed that this region was generally conserved between *M. pennsylvanicus* and *M. ochrogaster*, though some CpG sites differed (Figure S1). The subsequent analysis targeted the CpG sites found in *M. pennsylvanicus*. Alignments among house mouse *Mus musculus*, *M. ochrogaster* and *M. pennsylvanicus* were made using Clustal Omega with default parameters (Sievers et al., 2011). To differentiate between methylated and unmethylated CG sites in *M. pennsylvanicus*, 600 ng of DNA per sample was bisulphate converted (EpiTect Bisulfite Kit, Qiagen). Biotinylated PCR products were amplified using outer and nested primers (Table S1) targeting the CpG sites near the presumed Egr-1 binding site in the putative GnRH promoter. Outer primers were designed with MethPrimer (Li & Dahiya, 2002) and nested primers were designed with Pyromark assay design software. Biotinylated PCR products were run on an agarose gel and purified by gel extraction (QIAquick Gel Extraction Kit, Qiagen). Pyrosequencing was performed using a Pyromark Q106 ID pyrosequencer with samples run in duplicates. CpG methylation levels were quantified using Pyromark Q-CpG 1.0.9 software.

2.8 | Statistical analyses

Data analysis was conducted using R version 3.6.3 (R Core Team, 2020) and models were built using the packages NLME (Pinhiero et al., 2020) and LME4 (Bates et al., 2015). Figures were made using GGPLOT2 (Wickham, 2016).

To assess the proportion of lactating adult females at high and low density, juveniles were excluded from the dataset (animals <20 g).

A GLMM with a binomial family error distribution and a logit link function was used. The fixed effects included were density treatment, month, and the treatment and month interaction. Animal ID was included as the random effect to account for repeated measures, nested in year. To determine the significance of each of the fixed effects, the Wald chi-squared test was used. To estimate the effect of density treatment on sexual maturity, a GLMM with a binomial family error distribution and a logit link function was used. The fixed effects included were density treatment, body mass (g), the treatment and mass interaction, and sex. Animal ID was again included as the random effect to account for repeated measures and nested within year. To determine the significance of each of the fixed effects, the Wald chi-squared test was used. The average weight at sexual maturity for the population was determined as the mass when the probability of being sexually mature was 0.5 based on the logistic regression (the first weight at which half of the population is sexually mature).

For the FAM data, each reproductive class (reproductive or non-reproductive) was analysed using linear mixed models (LMMs) with treatment and sex as fixed effects and year as a random effect. Most animals provided a single faecal sample across the study, though a minority provided multiple samples (maximum of four samples). Because the majority provided a single sample, we did not use animal ID as a random effect and instead randomly dropped excess points from resampled animals.

To assess the effect of density treatment on relative gene expression, a GLMM with a Gamma distribution was used. Treatment, sex and body mass were included as fixed effects and year as a random effect. The treatment and sex interaction effect was originally included, but was found to be negligible, and removed from the model. To assess the effect of density treatment on methylation at each CpG site, LMMs with treatment and body mass as fixed effects and year as a random effect were used. Sexes were analysed independently, as females and males were bisulphite converted in two separate runs, and therefore were not able to be directly compared due to the potential for unequal conversion efficiencies across different runs.

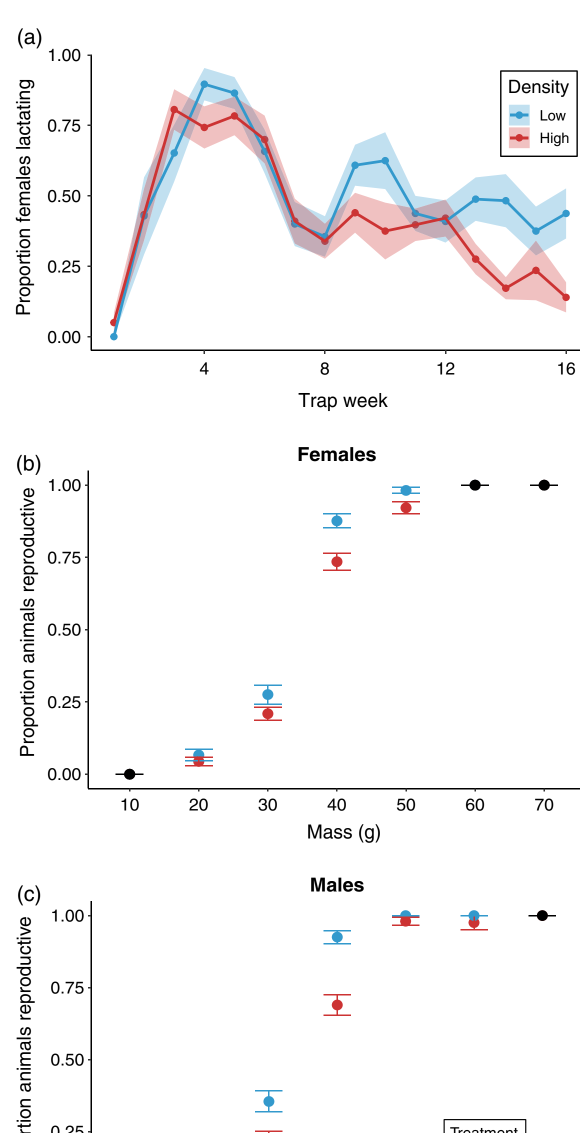

FIGURE 2 Reproductive performance of meadow voles from high-density and low-density experimental enclosures. (a) Proportion of lactating female voles at high density and low density across the summer breeding season. Points represent the treatment average (all enclosures pooled over the 3 years of the study 2016–2018) and ribbons represent ±*SE*. Week 1 is the first week following the release of animals into the enclosures (early May) and week 16 is the last trapping week of the summer (late August). The peaks reflect multiple litters across the summer; 3 in the low-density enclosures; ~2 in the high-density enclosures. (b) Proportion of reproductive animals in the high- and low-density enclosures by body mass for females and (c) for males. Body mass is binned in intervals of 10 g, black points represent where the treatments entirely overlap

3 | RESULTS

In total, we live-trapped 1,136 unique animals throughout the course of the study. On average, individuals were recaptured three times across the study period (maximum recaptures = 15, minimum recaptures = 1). This resulted in 3,324 data points used for the demography data. Reproductive performance differed at high and low density, with reduced reproduction at high density, analogous to the changes seen in cycling vole populations during the peak and decline. In the high-density enclosures, a lower proportion of adult (>20 g) females were lactating ($\chi^2 = 7.07$, $p < 0.01$) and this was more pronounced later in the breeding season (treatment and month interaction: $\chi^2 = 16.01$, $p < 0.01$; Figure 2a). Additionally, a lower proportion of voles were reproductive/sexually mature in both sexes at high density ($\chi^2 = 29.79$, $p < 0.001$). At low density, females were more likely to be sexually mature ($\chi^2 = 16.24$, $p < 0.001$; Figure 2b), but there was no significant interaction effect of mass by density ($\chi^2 = 2.5$, $p = 0.11$). The average mass at sexual maturity was 28.7 g at low density versus 32.2 g at high density. In males, there was a

main effect of treatment ($\chi^2 = 12.27$, $p < 0.001$) and a treatment and mass interaction effect ($\chi^2 = 7.08$, $p < 0.001$) on sexual maturity. The weight at which males became sexually mature was lower at low density (27.5 g at low density vs. 32.6 g at high density; Figure 2c). Thus, females were more likely to be mature at low density independent of body mass, and males were more likely to be mature at low density due maturation at an earlier mass.

We compared FAMs in each reproductive class (reproductive or non-reproductive) by treatment (Figure 3), accounting for sex as a fixed effect and year as a random effect. In reproductive animals ($N = 85$), there was a marginal effect of density, with low-density animals having higher FAM levels ($\beta = 0.19 \pm 0.12$, $t_{81} = 1.82$, $p = 0.07$). There was no effect of sex (males relative to females as the intercept: $\beta = -0.08 \pm 0.10$, $t_{81} = -0.83$, $p = 0.41$). In non-reproductive animals ($N = 66$), there was again a marginal effect of density, with low-density animals having higher FAM levels ($\beta = 0.30 \pm 0.17$, $t_{62} = 1.76$, $p = 0.08$) and no effect of sex (relative to females as the intercept: $\beta = -0.21 \pm 0.14$, $t_{62} = -1.47$, $p = 0.15$).

In the brain tissue samples collected from 67 juveniles (44 females and 27 males), we compared hypothalamic GnRH mRNA expression levels with treatment, sex and body mass as fixed effects and accounted for year as a random effect. Hypothalamic GnRH expression was lower in animals collected from high-density enclosures ($\beta = 0.3 \pm 0.2$, $t_{63} = -2.02$, $p < 0.05$; Figure 4a), in agreement with our findings of lower reproductive activity at high density. There was also an effect of sex, with male juveniles tending to have higher GnRH expression than females ($\beta = -0.51 \pm 0.15$, $t_{63} = -3.31$, $p < 0.001$). A potential explanation for the reduced GnRH could have been a smaller body size at high density, but there was no effect of body mass on GnRH expression ($\beta = 0.02 \pm 0.02$, $t_{63} = 1.39$, $p = 0.16$). Body mass also did not differ by density treatment ($\beta = 0.88 \pm 1.40$, $t_{63} = 0.63$, $p = 0.53$; Figure 4b). Thus, this GnRH change was not driven by differing body masses. There were no density treatment differences in ERα expression levels in either the hypothalamus ($t_{63} = 0.51$, $p = 0.61$) or the medial amygdala ($t_{61} = -0.58$, $p = 0.56$; Figure S2).

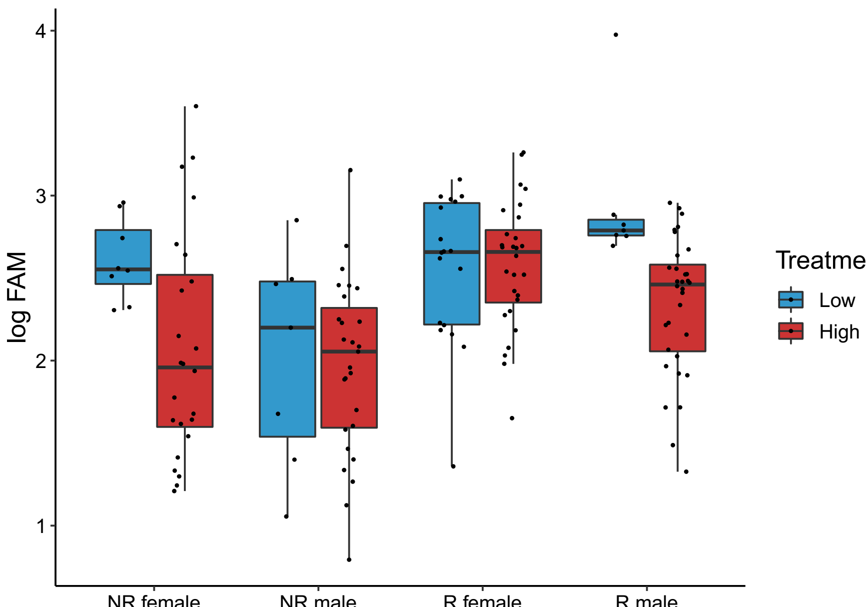

FIGURE 3 Log faecal androgen metabolite (FAM) levels compared between treatments among non-reproductive (NR) females and males, and reproductive (R) females and males. In both reproductive classes, there was a marginal effect of population density on FAM levels, with a decrease in FAM at high density in non-reproductive animals ($p = 0.08$), as well as in reproductive animals ($p = 0.07$)

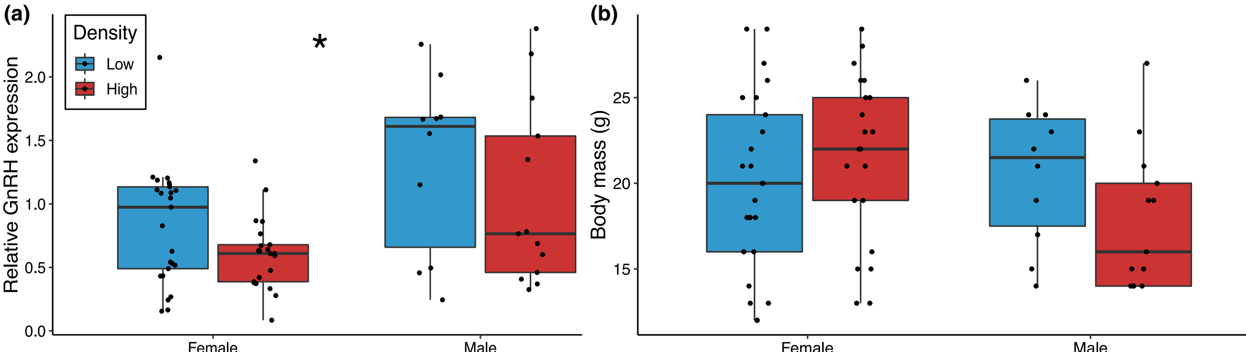

FIGURE 4 Hypothalamic GnRH gene expression (mRNA) and body masses of juvenile meadow voles collected from high-density and low-density enclosures. (a) Relative GnRH expression ($2^{-\Delta CT}$) was lower in juveniles born in high density, with the main effect of density on GnRH levels *$p < 0.05$. GnRH expression was lower in females than males ($p < 0.001$). (b) Body masses (g) of the juveniles collected for GnRH expression analyses showed no detectable treatment differences

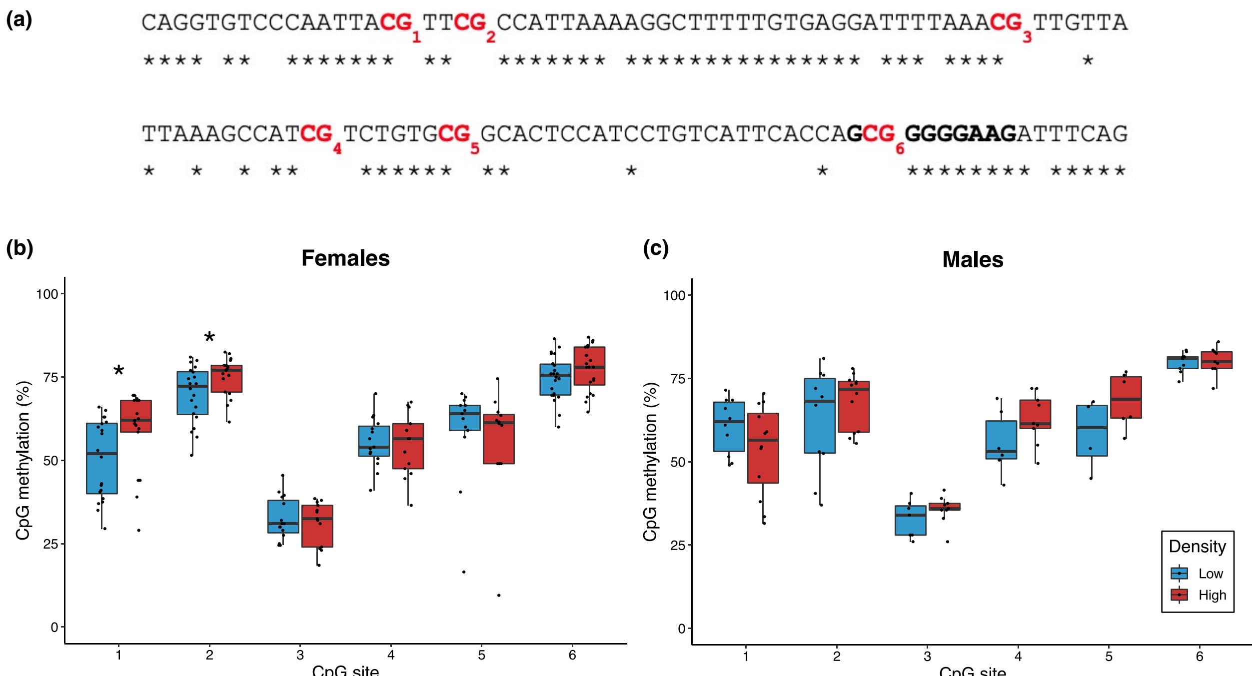

FIGURE 5 Meadow vole GnRH putative promoter region methylation in juvenile voles at high density and low density. (a) Meadow vole nucleotide sequence including the six targeted CpG sites in red. Asterisks indicate bases that are conserved with both the mouse and the prairie vole. The bolded region corresponds to the sequence of the proximal Egr-1 binding site in mice. (b) Female juvenile meadow vole CpG sites and percent methylation by density, with differences at sites 1 and 2 (*$p < 0.05$). (c) Male juvenile meadow vole CpG sites and percent methylation by density

We then tested whether this reduction in GnRH expression at high density was associated with epigenetic modifications to the GNRH1 promoter region. In meadow voles, there were five CpG sites directly upstream of the putative Egr-1 transcription factor binding site, and one CpG site within the putative Egr-1 transcription site (Figure 5a). Given the lower GnRH expression in high-density animals, we predicted that the percent methylation at some or all these CpG sites should be higher in high-density animals. We compared percent methylation by treatment, accounting for body mass again as a fixed effect, with year as a random effect. We found that female voles from high density had higher methylation levels at CpG site 1 ($\beta = -8.8 \pm 4.1$, $t_{32} = -2.15$, $p < 0.05$) and CpG site 2 ($\beta = -6.3 \pm 2.4$, $t_{32} = -2.67$, $p < 0.05$) and a marginal increase in methylation levels at CpG site 6 ($\beta = -3.5 \pm 2.0$, $t_{37} = -1.75$, $p = 0.09$; Figure 5b). Methylation was also negatively associated with body mass at CpG sites 2, 4 and 6, that is, larger females had lower methylation at these sites ($\beta = -0.52 \pm 0.23$, $t_{32} = -2.25$, $p < 0.05$; $\beta = -0.69 \pm 0.32$, $t_{23} = -2.15$, $p < 0.05$; and $\beta = -0.51 \pm 0.21$, $t_{37} = -2.42$, $p < 0.05$, respectively). Male meadow voles had no treatment differences at any of these CpG sites (all $p > 0.12$; Figure 5c) nor any effect of body mass (all $p > 0.26$).

4 | DISCUSSION

Our findings provide a potential mechanism for intrinsic regulation based on epigenetic and expression changes in a key regulatory gene. We found that high population density in meadow voles was associated with delayed sexual maturation, a lower proportion of breeding animals, marginally lower faecal androgen metabolite levels, lower hypothalamic GnRH expression in juveniles and higher levels of methylation at two CpG sites in the putative GnRH promoter in juvenile females. Significant increases in methylation occurred at only some, but not all, CpG sites tested, and this is consistent with epigenetic regulation of genes such as ESR1 and NR3C1 in laboratory rodents (Champagne et al., 2006; Weaver et al., 2004). Though demographic differences in reproduction across the phases of the vole population cycles have been previously described (Boonstra, 1989; Getz et al., 1979; Krebs et al., 1969; Norrdahl & Korpimäki, 2002), a physiological basis underlying these demographic changes has never before been demonstrated. By experimentally manipulating density within years, we show that these reproductive changes are driven by an intrinsic mechanism alone and not by environmental factors such as predation. Furthermore, changes in methylation levels at two CpG sites in females reveal the potential for stable, long-term differences in the reproductive axis induced by the natal environment. Thus, the repressed GnRH levels in female voles at high density may be epigenetically programmed, whereas the male reduction in GnRH levels may be an immediate response to social and environmental conditions. Because epigenetic effects are subject to maternal programming and/or other transgenerational effects, this may be a possible mechanism for the apparent heritability in reduced reproductivity that has previously been observed in meadow voles from the decline phase of population cycles (Mihok & Boonstra, 1992).

We designed this study to be as ecologically relevant as possible and to avoid experimental artefacts. Meadow voles were exposed to ecologically relevant population densities, based on prior work with this species at a location nearby in southern Ontario (Boonstra, 1989; Boonstra & Rodd, 1983). We trapped new founding populations each year of the study to avoid inbreeding and overgrazing/destruction of the vegetation within the enclosures. However, there are some caveats from our study that should be considered and that should guide future work. First, we examined two major reproductive-axis candidate genes, not the whole genome. It is likely that GnRH is not the only gene that is affected by population density in this species. There may be additive effects of several genes on reproduction at high and low density, and effects on other genes regulating other aspects of phenotype in differing density conditions (e.g. growth, behaviour). There are also other epigenetic mechanisms that may be involved aside from cytosine methylation, for example histone modifications. We do not suggest that methylation and expression changes in GnRH are the sole mechanism driving phenotypic changes in voles at high and low density, but rather that it is a likely mechanism and documented physiological change in response to population density. Furthermore, to understand the generality of these changes, naturally cycling populations of voles should be tested with the prediction that they should show the same changes in GnRH that we observe in the meadow vole enclosure populations.

The second caveat of these findings is the functionality of the causal chain we are proposing. With limited genomic resources and studies in meadow voles, we cannot directly demonstrate the functional significance of these CpG sites at present nor show that these changes in GnRH are directly responsible for inhibiting reproduction at high density. However, we believe these challenges should not prevent exploratory studies in ecologically relevant species. Despite the high variation inherent in ecological data, we were able to detect a signature of population density both in gene expression and in the female meadow vole epigenome. Future molecular work in voles could directly interrogate the function of these, and potentially other, changes in response to population density. Much work has focused on prairie vole neurobiology, and direct manipulation of neural gene expression has been conducted in prairie voles by viral insertion (e.g. Keebaugh & Young, 2011) and more recently by CRISPR/Cas9 knockout (Horie et al., 2019). These techniques will become more applicable to additional species as reference genomes and other resources develop. While the above studies have been focused on the neurobiology of pair-bonding in prairie voles, these molecular techniques have relevance for questions in ecology as well. Ultimately, functionality would need to be tested by observing the reproductive performance of these altered animals. Additionally, to interrogate the epigenetic component, methylation could be altered through the use of pharmacological agents (such as zebularine, e.g. Keller et al., 2018), to determine whether this could eliminate phenotypic differences between high-density and low-density animals. If that was the case, it would indicate that such differences between high and low-density populations are indeed epigenetically driven.

This work fits into a growing body of evidence demonstrating how population epigenetics shape animal behaviour, physiology and subsequently population processes. In the study most comparable to ours, Alvarado et al. found that social crowding in the early life environment affects GnRH1 promoter methylation in Burthon's mouthbrooder *Astatotilapia burtoni* cichlid fish (Alvarado et al., 2015). The result was the opposite of ours: they found decreased methylation in the promoter and increased GnRH expression with social crowding. However, because the *A. burtoni* study was conducted in a laboratory setting, and because it is unknown what these densities would correspond to in the wild, it is unclear whether this effect occurs in nature or if it has adaptive value in this species. Another study compared indicators of global DNA methylation in desert locusts *Schistocera gregarina* reared in crowded and uncrowded conditions (Mallon et al., 2016). This species shows two natural phenotypes that it can transition between a solitary phenotype and a gregarious phenotype (which exhibits swarming behaviour), and exposure to crowded conditions are thought to trigger transition to the latter. The authors found distinct differences in methylation patterns among solitary-reared locusts, crowd-reared locusts, and solitary-reared but exposed to acute crowding locusts, though the methods used could not identify the particular genes affected. Here, early life population density clearly altered the epigenome and phenotype or individuals in ecologically relevant ways. Finally, a study in prairie voles demonstrated that natural individual variation in DNA methylation in the enhancer region of the vasopressin 1a receptor (avpr1a) was associated with differences in avpr1a expression in the retrosplenial cortex of the brain. These avpr1 expression differences have been tied to degree of social monogamy in this species, which shows natural variation in social reproductive behaviour (Okhovat et al., 2017). Ultimately, these and other work, including our findings, demonstrate that DNA methylation differences are a fundamentally important mechanism that can alter how individuals interact within populations.

The changes we observed in GnRH at high population density in meadow voles have potentially extensive ecological consequences. Ecologists have been searching for a mechanism to explain vole and lemming population dynamics for a century (Elton, 1924), and though intrinsic regulation was proposed over 50 years ago (Chitty, 1967), it was based on the wrong mechanism—gene frequency changes—which was subsequently rejected (Boonstra & Boag, 1987; Boonstra & Hochachka, 1997). We demonstrate the suppression of GnRH, the regulator of the reproductive axis, as a direct consequence of population density, supporting the predictions of the intrinsic regulation hypothesis. It remains to be determined the degree to which our findings apply to non-cyclic species, but it was proposed that self-regulation in cyclic small mammals was simply a special instance of a general phenomenon (Chitty, 1967). Ultimately, one of the principal goals of animal ecology is to understand the factors which regulate and limit animal abundance (Krebs, 2020). We underscore that intrinsic, social regulation of reproductive physiology is one of these critical factors.

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AUTHORS' CONTRIBUTIONS

P.D.E. and R.B. designed the research and wrote the manuscript; P.D.E., C.F.-L. and R.B. collected the data; R.P. developed and provided the faecal metabolite assays, and all authors contributed to revising the manuscript and gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data associated with this publication are available on Figshare https://doi.org/10.6084/m9.figshare.13256918 Edwards (2020).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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