ARTIFICIALLY ADAPTIVE NEUROANATOMICAL SPECIALISATIONS IN DOMESTIC PIGEON (*COLUMBA LIVIA*) BREEDS

AUDREY ELIZABETH MARY GUYONNET
Bachelor of Environmental Sciences (Ecology), University of Guelph, 2020

A thesis submitted
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

NEUROSCIENCE

Department of Neuroscience
University of Lethbridge
LETHBRIDGE, ALBERTA, CANADA

ARTIFICIALLY ADAPTIVE NEUROANATOMICAL SPECIALISATIONS IN DOMESTIC PIGEON (*COLUMBA LIVIA*) BREEDS

AUDREY ELIZABETH MARY GUYONNET

Date of Defence: August 12, 2022

Dr. A. Iwaniuk Thesis Supervisor	Associate Professor	Ph.D.
Dr. S. Pellis Thesis Examination Committee Member	Professor	Ph.D.
Dr. I. Whishaw Thesis Examination Committee Member	Professor	Ph.D.
Dr. S. MacDougall-Shackleton External Examiner University of Western Ontario London, Ontario	Professor	Ph.D.
Dr. A. Luczak Chair, Thesis Examination Committee	Professor	Ph.D.

ABSTRACT

The domestication of wild Rock Pigeons (*Columba livia*) has formed many breeds unique in behaviour, such as homing in homing pigeons and sexual displaying in cropper pigeons. Specialised behaviours are likely facilitated by concomitant changes in regional brain anatomy. Here we test how selection for behaviour is associated with neuroanatomical variation by comparing hippocampal formation (HF) and septal volume and neuron number across domestic and feral pigeons. We found significant neuroanatomical variation among breeds. Cropper pigeons have larger septum volumes, whereas homing pigeons have larger HF volumes and relatively smaller septum volumes. Furthermore, homing pigeons have significantly more HF neurons than most breeds, and twice as many HF neurons as feral pigeons, a finding we attribute to selection for homing. Cropper pigeons have more HF and septal neurons than most other breeds. These results suggest artificial selection for behaviour might be accompanied by significant changes in neuron number and density.

ACKNOWLEDGEMENTS

Foremost, thank you to Dr. Andrew Iwaniuk for the opportunity to be a part of such an incredible project and lab. Throughout my time at the University of Lethbridge, Dr. Iwaniuk has aided me to improve valuable scientific skills, such as how to critically engage with science and how to assess and troubleshoot protocols at every step (I will still be able to run immuno trials from memory in 50 years). The opportunity to learn such a wide array of lab, field, and analytical techniques has been truly invaluable, and I am so thankful to have been a part of such an incredible team. And of course, thank you so much to Drs. Ian Whishaw and Sergio Pellis for their guidance and encouragement over the course of this project. Their knowledge and feedback have been essential to the creation of this final manuscript.

Next, I of course have to thank Kelsey Racicot, my accomplice in what at times felt like an insurmountable project. Kelsey and I first spoke in August of 2020, and it took a single phone conversation to know that whatever happened over the next two years, we'd be in it together. I truly would not have known where to start without her constant guidance and support. I'm so glad we got to attend ICN 2022 together, and think I speak for us both when I say navigating Lisbon on a handful of hours of sleep and more cappuccinos than should be legal is something we'll forever remember all too well.

Benjamin Brinkman, where do I even start? First, let me say that without Ben this project would be a short step from impossible, a two-year long prisoner-hat dilemma, or six pigeons in the back of a pickup with no one to let us into the Boneyard. Ben has been an incredible supporter of every aspect of this project and working with him has been truly delightful. I have enjoyed every minute we've spent joking together in the lab – even when the references were too

obscure for anyone else, and especially when they were too obscure for one of us. It's been pretty bumper cars if you ask me.

Thank you to all the summer students for their help with this project: Sydney Irvine, Jocelyn Kaloa, and Grace Lake. Thank you to my friends and family for their endless support, and for the late night and weekend phone conversations that kept me endlessly entertained as I counted cells. Finally, thank you to everyone else who had a part in supporting me over the course of this project, it would not have been possible without you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANCOVA – analysis of covariance
ANOVA – analysis of variance
APH – area parahippocampalis
CB – cerebellum
CCAC – Canadian Council on Animal Care
CDL – dorsolateral corticoid area
ChAT – choline acetyltransferase
CI – confidence interval
df – degrees of freedom
F – F-ratio
FITC – fluorescin 5-isothiocyanate
g – grams
HF – hippocampal formation
Hp – hippocampus
HSD – honestly significant difference
IEG – immediate early gene
LRP8 – low-density lipoprotein receptor 8
LS – lateral septum
LV – lateral ventricle
mm^3 – millimetres cubed
MS – medial septum
n – sample size
NeuN – neuronal nuclei
p – p-value
PFA – paraformaldehyde
PBS – phosphate buffered saline
PBST – phosphate buffered saline + triton
r – correlation coefficient
ROI – region of interest
SD – standard deviation
T – T-value

CHAPTER ONE: GENERAL INTRODUCTION

For over 30,000 years, humans have selectively bred animals for specific traits, a process called domestication (Kruska 1988, Francis 2015). Domestication has several unique conditions: 1) unconscious or goal-oriented artificial selective breeding; 2) management practices that compensate any potential fitness losses; and 3) the animal's necessities (food, shelter) are provisioned to reduce resource competition (Price 1984, Daniels and Bekoff 1989). Domestic animals are no longer subject to most natural selective pressures, and artificial selection acts as a surrogate force driving the variability of traits. Instead of conforming to natural ecological niches, as have their ancestors, domestic animals fill select "performance" niches, excelling in physical ability (speed, endurance, strength), commodity production (food, oil, leather), and/or exaggerated morphological characters (colour patterning, size) (Kruska 1988). Artificial selection pressures have not remained static and have changed with the evolving roles of animals in society. Since the industrial revolution, the use of domestic animals in the labour and military sectors has diminished, changing the focus of selection to production, competition, and companionship. Over time, differential selective breeding has created variation not only among domestics and their ancestor species, but also among breeds.

Despite varying selective pressures across domesticated species, all of them have been selected for tameness (Kruska 1988). Tame animals, in addition to getting along with people, exhibit higher tolerance to biological and/or physical stressors. While captive living has few natural external threats, such as predation or resource scarcity, selection for tameness can limit the effects of novel manmade stressors, e.g., high density herd-living and frequent human contact (Price 1984). The creation of truly 'tame' animals follows an ongoing generational selection process and is only relaxed under circumstances where domestics are accidentally released back

into the wild (Francis 2015). In such instances, domestic animals undergo feralization (Price 1984, Daniels and Bekoff 1989). Feral animals are desocialized from humans, can display behaviours similar to wild animals, and will often interbreed with wild conspecifics (Francis 2015). This means that the existence of truly 'wild' versions of domestic species have become increasingly unlikely. Despite this, comparisons among domestic animal breeds can reveal how differential artificial selection can cause lasting changes to the behaviour and morphology of species.

The Rock Pigeon (*Columba livia*) is a remarkable example of intraspecific variation produced by artificial selection. Rock Pigeons were first domesticated as early as the Pleistocene epoch (~10,000 years ago) in the Fertile Crescent of the Middle East (Shapiro and Domyan 2013). Pigeons now coexist with humans on every continent with the exception of Antarctica. Until the 19th-century, domestic pigeon breeds were assumed to be descendant from several distinct ancestral lineages (Darwin 1859, 1894, Gilbert and Shapiro 2013). Charles Darwin was one of the first to document that interbreeding any combination of pigeon breeds resulted in a reversion to a common, presumably ancestral, phenotype (Darwin 1859). Darwin then hypothesised that feral pigeons were descendant of escaped domestics, and that all pigeons shared a common ancestor in the Rock Pigeon (Darwin 1859, 1894). Like other domestic species, the morphological and behavioural diversity of pigeons can be attributed to thousands of years of intensive artificial selection (Gilbert and Shapiro 2013).

The largest period of pigeon breed differentiation began in the 16th century in the Middle East and Southern Asia. By the 17th century, Europeans had taken up pigeon breeding as a popular hobby, creating more standardised qualifications for incorporation into breed registries (Shapiro and Domyan 2013). Due to divergent selection, there are now about 350 breeds of

pigeon that vary in size from 200 g (Valencian figurita) to >1000 g (giant runt). The colour patterning among- and within-breeds is extensive, and Catalonian tumblers alone exhibit 318 colour combinations. Flight pattern can vary as highflyers can remain airborne for many hours whereas parlour rollers are completely flightless (Baptista and Horblit 2009). This variation has resulted in breeds classified into three functional subgroups: show, utility, and sporting. Show pigeons include breeds selected for colour patterning, feather type, or other distinct morphological characters, e.g., Norwich cropper, Old Dutch capuchine. Utility pigeons are bred for their meat, or squab, which is harvested from young commercially raised birds, e.g., king pigeon. Sporting pigeons are selected for high performance traits, such as acrobatic flying techniques, endurance, and/or flight speed, e.g., roller, homing pigeon. As a result of the selection and development of many breeds, the pigeon exhibits more physical and behavioural trait variation than any other single avian species (Price 2002).

Of all pigeon breeds, the homing pigeon is perhaps one of the most distinctive examples of artificial selection for behaviour (Jerolmack 2007, Gilbert and Shapiro 2013). Homing is a navigational behaviour in which an animal returns to a home nest after foraging sessions. Homing pigeons do so by integrating multisensory cues to orient towards their home loft, often by the most direct route (Gallistel 1990, Shapiro and Domyan 2013). For wild Rock Pigeons, homing is used to locate a cliffside nest up to 80 kilometres away in a stark landscape with few visual cues, e.g., ocean, desert (Alleva et al. 1975). Artificial selection for the homing behaviour began as early as 3,000 BC (Shapiro and Domyan 2013), and modern homing pigeons can locate a home loft from a novel location as far as 1,000 kilometres away (Strasser et al. 1998, Jacobs 2003, Mehlhorn and Rehkamper 2009, Shapiro and Domyan 2013). As juveniles, homing pigeons familiarise themselves with odour, visual, magnetic, and sun compass cues at their home

loft (Strasser et al. 1998, Jacobs 2003). Adult pigeons released into unfamiliar locations then return home using combined information from these familiarised cues (Strasser et al. 1998, Bingman 2018). Despite generations of improvements to the homing behaviour, little is known about whether this behavioural selection has resulted in changes to brain anatomy.

Domestication is typically accompanied by significant changes to the size, composition, and shape of the brain (Kruska 1988). Often, domestic breeds have relatively small brains, and brain regions, compared with their wild ancestors (Kruska 1988, Agnvall et al. 2017). This trend, summarised as the 'regression hypothesis' (Rehkamper et al. 2008), is proposed to be due to a decreased reliance in captive animals on complex foraging and predator detection/evasion behaviours (Agnvall et al. 2017). As these behaviours are expressed less frequently, or even lost entirely, it is possible that their associated brain regions have decreased in size accordingly (Jerison 1973). However, volume is not the only metric of neuroanatomy. Other features including neuron number, density, and survivorship may also be correlated to cognitive ability (Sherry et al. 1992). Furthermore, not all behaviours have been supressed by the selection process, as is demonstrated by the improved homing ability of pigeons. This improvement in spatial navigation may therefore be dependent upon localised neuroanatomical changes.

Many brain regions are involved in homing, but a key region that has been the focus of decades of research is the hippocampal formation (HF) (Bingman et al. 1990, Strasser et al. 1998, Ben-Yishay et al. 2021). The avian HF consists of both hippocampus (Hp) and area parahippocampalis (APH) (Atoji and Wild 2006) and is involved in many aspects of spatial cognition (Sherry 2006, Pravosudov and Roth 2013, Herold et al. 2015). APH is most active during homing (Shimizu et al. 2004), but most homing pigeon studies have often focused on Hp alone. The Hp of homers is larger than it is in wild Rock Pigeons (Rehkamper et al. 1988,

Rehkamper et al. 2008, Shapiro and Domyan 2013), but no different in size from other domestic breeds that are not selected for homing (Ebinger and Löhmer 1984, Rehkamper et al. 2008). Several problems arise, however, when correlating spatial abilities or spatial cognition with HF volume. For example, volume can be correlated with factors that are not spatially dependent, such as exercise (Uysal et al. 2005, Erickson et al. 2011). Pigeon breeds are often housed differently depending on their purpose: show and utility breeds are housed in cages or aviaries, whereas sporting breeds are housed in aviaries with daily access to several hours of free flight. Therefore, it is possible that either artificial selection for behaviour or environmental conditions could influence HF neuroanatomy. Furthermore, neuron number, rather than region volume, is considered by several authors to be a better measure of cognitive ability in birds (Roth et al. 2010, Pravosudov and Roth 2013). An appropriate comparison of pigeon breeds should therefore include neuron numbers as well as brain region volumes.

While HF is proposed to be important for spatial navigation (Pravosudov and Roth 2013, Herold et al. 2015, Ben-Yishay et al. 2021), it also has other functions. HF, amygdala, hypothalamus, and septum make up the limbic system that mediates emotional response behaviours, such as aggression, reproduction, and fight-or-flight responses. These roles in courtship and agonistic behaviours could mean that birds selected for elaborate sexual displays (Norwich cropper pigeons) or birds that show increased aggression (feral pigeons) might also differ in HF neuroanatomy from other breeds. Furthermore, other structures in the limbic system, such as the septum, are highly connected to Hp and APH (Atoji and Wild 2006) and play a role in spatial working memory processes (Lubar and Numan 1973, Peterson and Bingman 2011, Coppola et al. 2021) that could be important for goal-oriented navigation. It is therefore

important to not only examine HF anatomy in relation to homing and other selected behaviours, but also the septum and, ultimately, other structures within the limbic system.

The adaptive specialisation theory suggests that differences in behavioural complexity are correlated with changes to the brain regions that control them, e.g., region volume, neuron number, neuron size (Sherry et al. 1992, Roth et al. 2010). It is possible that homing pigeon HF and associated structures differ from other breeds due to the spatial demands of this behaviour. Alternatively, HF and/or septum could differ in breeds with marked differences in courtship or antagonistic behaviours, such as Norwich croppers or feral pigeons. This thesis addresses these alternatives by measuring brain region volume, neuron number, and neuron density across a variety of domestic and feral pigeons. In doing so, we will determine: 1) if selection for the homing behaviour has driven changes in hippocampal neuroanatomy in homing pigeons; and 2) if there are changes in the septum of pigeons associated with selection for spatial navigation or other behaviours. Ultimately, this research is important for demonstrating the extent to which artificial selection can alter brain anatomy in a similar fashion to what has already been documented in wild populations exposed to natural selection factors.

CHAPTER TWO: HAS ARTIFICIAL SELECTION FOR SPATIAL COGNITION CHANGED HIPPOCAMPAL FORMATION ANATOMY IN THE DOMESTIC PIGEON (*COLUMBA LIVIA*)?

INTRODUCTION

Domestic species are artificially selected for possession of desired behavioural, morphological, or production traits (Kruska 1988, Francis 2015). While production traits often differ among species (e.g., milk yield, egg production, body size), all species are selected for ease of handling and/or tameness. Tame animals exhibit lower fear responses and adapt better to herd-living, making them easier to house at a larger scale (Price 1984, Kruska 1988). Alongside selection for tameness, many domestic species have smaller brains in comparison to their wild ancestors (Kruska 1988). The 'regression hypothesis' proposes that this reduction results from a simplification, or even loss, of behaviours in captivity (Rehkamper et al. 2008). Captive environments are strictly managed by humans, meaning some behaviours are no longer essential to survival and reproduction, such as complex predator evasion and foraging strategies. However, not all behaviours have been supressed by the selection process, and some have been improved in specific breeds, such as singing in domesticated songbirds (Okanoya 2004, O'Rourke et al. 2021) and spatial navigation in homing pigeons (*Columba livia*) (Edrich and Keeton 1977).

Homing pigeons are one of the most famous examples of artificial selection for a behavioural trait in a domestic species. 'Homing' is the ability of pigeons to navigate unfamiliar terrain back to a home loft by combining information from novel and learned sensory inputs (Strasser et al. 1998, Jacobs 2003, Mehlhorn and Rehkamper 2009, Shapiro and Domyan 2013). By conducting multiple experimental flights as juveniles, homing pigeons familiarise themselves

with orientation cues at their home loft, such as sun azimuth, magnetic polarity, visual landmarks, and odours (Strasser et al. 1998, Jacobs 2003). Adults released in unfamiliar locations use a two-part 'map and compass' model to return home: 1) the 'map' is used to determine their relative location based on a combination of odour and visual cues; and 2) the 'compass' allows continuous path readjustment by means of magnetic field and sun compass inputs (Strasser et al. 1998, Bingman 2018). While wild Rock Pigeons use homing to navigate between nesting and foraging sites as far as 80 km apart (Alleva et al. 1975), this range has been increased to approximately 1,000 km in domestic homing pigeons (Degner and Blechman 2011, Gilbert and Shapiro 2013, Shapiro and Domyan 2013). The ability of homing pigeons to navigate such long distances from unfamiliar locations is attributed to improvements to spatial cognition caused by artificial selective breeding practices.

In birds, spatial cognition is thought to be mediated by the hippocampal formation (HF), a telencephalic region that encompasses both hippocampus proper (Hp) and area parahippocampalis (APH) (Atoji and Wild 2006, Herold et al. 2015), which together are analogous to the mammalian Hp (O'keefe and Nadel 1978, Sherry et al. 1992). Not only is the avian HF associated with performance in spatial tasks (Sherry 2006), it also appears to play a significant role in homing behaviour. In inexperienced homing pigeons, HF lesions compromise an individual's ability to learn a navigational map and find homeward bearing (Strasser et al. 1998, Bingman et al. 2005). In experienced homing pigeons, HF lesions impair familiar landmark recognition and increase navigation duration and path circuitousness (Bingman et al. 1990, Bingman et al. 2005). Further, HF activity increases in relation to preferred directional bearing during routine navigation (Ben-Yishay et al. 2021) and during homing (Shimizu et al. 2004, Epstein et al. 2017). Overall, homing is at least partially dependent on the HF (Herold et

al. 2014). Given the importance of HF in homing, one might expect corresponding changes in the size of HF (Jerison 1973, Sherry 2006) in homing pigeons, but evidence for such an enlargement remains mixed.

The adaptive specialisation theory suggests differences in behavioural complexity cause corresponding changes to the brain regions that control them (Sherry et al. 1992). While homing pigeon Hp is reported to be larger than that of "wild" Rock Pigeons (Rehkamper et al. 1988, Rehkamper et al. 2008, Shapiro and Domyan 2013), it is not larger than that of several other domestic breeds (Ebinger and Löhmer 1984, Rehkamper et al. 2008). However, these comparisons are problematic for several reasons. First, previous comparisons measured Hp only, but APH is more active during homing (Shimizu et al. 2004), making overall HF volume a more appropriate measure of spatial cognition (Atoji and Wild 2006). Measuring HF also avoids some of the problems associated in defining avian homologues to the mammalian dentate gyrus, subiculum, and entorhinal cortex (Striedter 2015). Second, Rehkamper et al. (1988, 2008) used body mass as a scaling variable, but body mass can be selected for independently of behaviour. Instead, telencephalon volume is more relevant and consistent with other literature on avian HF (Roth et al. 2010, Ward et al. 2012). Last, a sole focus on volume neglects other aspects of neuroanatomy related to spatial cognition (Pravosudov and Clayton 2002). Studies of food caching species indicate that birds with greater cache-recovery performance have more and larger HF neurons in both field (Pravosudov and Clayton 2002, Freas et al. 2013) and lab-based experiments (Gould et al. 2013) as well as higher rates of neurogenesis and neuron survivorship (Sherry 2006, Chancellor et al. 2011). Neuron number and/or density have even been suggested to be more appropriate proxies of cognitive processing capacity than HF volume: increases in neuron number aid to better integrate neural networks (Roth et al. 2010) and predict improved

performance at correlated behavioural tasks (Gould et al. 2013, Pravosudov and Roth 2013, Kverkova et al. 2022). However, HF neuroanatomy can also be influenced by numerous other factors, like activity level (Healy et al. 1996, Day et al. 2008, Roth et al. 2010, Pravosudov and Roth 2013), which may differ across pigeon breeds. A full analysis of domestic pigeon HF anatomy should therefore compare not only volume, but also neuron number, across both sporting and show breeds that vary in behaviour and selection regime (*Figure 2.1A-E*).

Here, we specifically test if experienced homing pigeons differ in HF anatomy from other breeds. We compare homing pigeons (*Figure 2.1E*) with three other groups of pigeons: show, sporting, and feral. Show breeds (*Figure 2.1B* and *C*) are selected for posture, plumage, size, and in the case of the Norwich cropper, exaggerated sexual characteristics (enlarged crop, upright body posture) (Darwin 1894). Show breeds do not fly outside of an aviary or loft and therefore have no experience navigating outside of their immediate captive environment. Sporting breeds are selected for endurance and/or inflight behaviours, like 'tumbling' (aerial somersaults) in roller pigeons (*Figure 2.1D*) (Mowrer 1940). Finally, feral pigeon (*Figure 2.1A*) populations are comprised mostly of homing pigeons lost during racing events, in addition to some other escaped domestic breeds, but no longer experience artificial selection. Instead, they are subject to natural selection pressures, including predation, variable weather conditions, and food availability.

Here, we provide a more effective test of whether HF anatomy differs in the homing pigeon compared with other breeds. Expanding upon previous findings, we analyse both volume (Hp, APH, HF, and telencephalon) and neuron number (HF) across homing, show, sporting, and feral pigeons. Based on what we know about the involvement of HF in homing processes (Pravosudov and Clayton 2002, Jacobs 2003, Bingman et al. 2005, Gould et al. 2013) and the intense selection that homing pigeons have been under, we predict that homing pigeons will have

relatively larger HFs that contain more neurons than other breeds. Activity level can also significantly influence HF volume (Healy et al. 1996, Day et al. 2008, Roth et al. 2010, Pravosudov and Roth 2013), so we predict that inactive show breeds will have fewer HF neurons in comparison to active sporting breeds. Finally, stress may also negatively influence HF neurogenesis and survival (Sherry and MacDougall-Shackleton 2015, Smulders 2017). Since feral pigeons likely experience high stress from living in urban environments, we also predict that they might have fewer HF neurons than domestic pigeon breeds.

MATERIALS AND METHODS

Animals

A total of 51 male and female domestic pigeons were used, including feral pigeons (n = 8), homing pigeons (n = 10), sporting breeds (rollers, n = 6; highflyers, n = 7), and show breeds (capuchines, n = 4; Norwich croppers, n = 5; American show homers, n = 5; American show rollers, n = 6). All homing pigeons were trained and used in local races. All other breeds were sourced from pigeon breeders across Alberta and Saskatchewan. Feral pigeons were trapped using standard wire baited traps in Lethbridge and Hannah, Alberta. Birds were immediately weighed (Table 2.1) and sacrificed using an intracoelomic injection of sodium pentobarbital (2 mL/kg body weight) before being perfused transcardially with 0.1 M phosphate buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde (PFA). The brains and eyes were removed and stored in 4% PFA at 4 ^{o}C for 1 to 2 weeks. Brains were weighed (Table 2.1), cryoprotected in 30% sucrose PBS solution, moved into antifreeze, and stored at -20 ^{o}C until embedding. All animals were handled according to Canadian Council on Animal Care (CCAC) guidelines and animal welfare policies at the University of Lethbridge (Animal Welfare Protocol #2011).

Histology

Before histological processing, the cerebella were first removed and stored for future projects. Brains were then embedded in gelatin blocks and sectioned coronally on a freezing stage microtome at 40 µm thickness. All sections were collected in PBS + 1% sodium azide solution in multi-well plates. For all specimens, every fourth section (1:4 series) was mounted onto gelatinised slides. Once dry, sections were washed in chloroform and stained for Nissl bodies using thionin acetate followed by a graded ethanol series. Sections were cleared in Hemo-De (Thermo Fisher Scientific, HD150A) and coverslipped with Permount (Thermo Fisher Scientific, #SP15500).

Eighteen to 22 sections equally spaced throughout the rostrocaudal extent of HF were immunolabeled for the neuron specific antigen, NeuN (Cao et al. 2002). Sections were rinsed in 1% PBS (pH 7.4), then incubated in 10% normal goat serum (Jackson ImmunoResearch, 005-000-121) at a 1:10 dilution in 1% PBS + 0.025M Triton (PBST) for 1 hour. The sections were then incubated in a monoclonal mouse anti-NeuN primary antibody (clone A60, Sigma Aldrich, MAB377) at a 1:1000 dilution in 0.0125M PBST on a shaker plate for 24 hours at room temperature. The sections were rinsed again in PBS and then incubated for 4 hours in fluorescein (FITC) goat anti-mouse secondary antibody (Jackson ImmunoResearch, 111-095-144) at a 1:200 dilution in 0.0125M PBST on a shaker plate at room temperature. Finally, the sections were rinsed and mounted onto gelatinised slides.

Stereology

All volumetric measurements and neuron counts were made using unbiased stereology (Howard and Reed 2004). Regions of interest (total brain, telencephalon, hippocampal formation, hippocampus proper, and area parahippocampalis) were differentiated according to

multiple stereotaxic atlases of bird brains (Karten and Hodos 1967, den Boer-Visser et al. 2004, Puelles et al. 2018) (*Figure 2.2*). Volumetric measurements were made on a Zeiss Axio Imager M2 microscope using the Cavalieri Estimator Probe in StereoInvestigator™ with a 400 µm grid size for all regions of interest. Quantifications for total brain and telencephalon were taken using a 1x objective lens; all other regions were quantified using a 2.5x objective lens (Table 2.2). Coefficients of error (Gunderson, m = 1) for all volumes were < 0.05 with a 95% confidence interval (CI) < 0.06 for all specimens.

The total number of neurons within HF were estimated using the Optical Fractionator method (Altunkaynak et al. 2011), as implemented in StereoInvestigator™. The following parameters were used: a grid spacing of 650 µm, a grid size of 40 µm, a dissector zone of 15 µm, and upper and lower guard zones of 5 µm. Neuron counts were completed using a 40x immersion oil lens on a Zeiss Axio Imager M2 microscope. Neurons labelled with NeuN (*Figure 2.3*) were counted if at least two-thirds of the cell membrane was visible and at least two-thirds of the neuron came into focus within the dissector (Table 2.3). Coefficients of error (Gunderson, m = 1) for all cell counts were < 0.10 with a 95% CI < 0.10 for all specimens.

Statistical Analyses

All statistical analyses were performed in Jamovi, an open-source statistics software built using R statistical language (The jamovi project 2022). Only birds from which both volume and neuron counts were measured were included in the analyses. Absolute values were analysed using Fisher's one-way analysis of variance (ANOVA) and a Tukey's honestly significant difference (HSD) post-hoc test. Relative values were analysed using analyses of covariance (ANCOVAs) of log-transformed data and Tukey's HSD post-hoc tests. Covariates included body mass, whole brain volume (minus region of interest (ROI)), or telencephalon volume (minus

ROI). Interaction effects (breed x covariate) were not significant for all variables, indicating no significant differences in slope among breeds. Interaction effects were therefore removed from final ANCOVA models.

RESULTS

Hp, APH, and HF volumes

Analyses of absolute data showed Hp and HF, but not APH, volume differed significantly among breeds (Table 2.4). Homing pigeons had the largest HF (mean = 64.2 mm^3) and Hp (mean = 38.7 mm^3) volumes, whereas rollers had the smallest HF volumes (mean = 51.0 mm^3) and highflyers had the smallest Hp volumes (mean = 30.4 mm^3). Rollers had the largest APH volumes (mean = 26.9 mm^3) and capuchines had the smallest APH volumes (mean = 20.1 mm^3). A Tukey's HSD post-hoc test revealed homers had significantly larger Hp volumes than highflyers and rollers, and significantly larger HF volumes than highflyers alone (*Figure 2.4A*). There were no significant differences among any other breeds.

Relative to telencephalon (minus ROI) volume, Hp, APH, and HF volumes differed significantly among breeds (Table 2.5). Despite the overall model being significant, a Tukey's HSD post-hoc test revealed no significant interbreed differences in APH and Hp volume. In the analysis of HF, homing pigeons had significantly larger HF volumes than highflyers, but no other significant differences were detected among breeds. However, as shown in *Figure 2.4B*, homing pigeons tend to have larger relative HF volumes compared to most other pigeons.

HF neuron number and density

The absolute number of HF neurons differed significantly among breeds (Table 2.4). Norwich cropper and homing pigeons had the highest neuron counts (both means = 2.4 x 10^6),

and feral pigeons had the lowest neuron counts, less than half of that of homing pigeons ($mean_F = 1.1 \times 10^6$) (*Figure 2.5A*). Post-hoc tests revealed that homing pigeons and Norwich croppers had significantly more neurons than feral, highflyer, roller, and capuchine pigeons. Conversely, feral pigeons had significantly fewer neurons than homing, show roller, show homer, and Norwich cropper pigeons.

HF neuron number relative to HF volume also differed significantly among breeds (Table 2.5). Homing pigeons and croppers had significantly more neurons, relative to HF volume, than feral, highflyer, roller, and capuchine pigeons whereas feral pigeons had significantly fewer neurons than homing, highflyer, show roller, show homer, and cropper pigeons. *Figure 2.5B* demonstrates that non-homing sporting breeds (highflyers and rollers) trend along the whole-population average, feral pigeons trend below average, and homing pigeons are near-universally above average.

HF neuron density, calculated by dividing neuron numbers by HF volume, also differed significantly among breeds (Table 2.4). Norwich cropper and homing pigeons had the highest HF neuron densities ($mean_{NC} = 4.0 \times 10^4$ neurons/mm^3; $mean_H = 3.8 \times 10^4$ neurons/mm^3), and feral pigeons had the lowest neuron density, again less than half of that of homing pigeons ($mean_F = 1.9 \times 10^4$ neurons/mm^3) (*Figure 2.5C*). These differences in HF neuron number and density between homing and feral pigeons are even apparent when looking at the sections. As shown in *Figure 2.3*, homing pigeons have far more neurons labeled than feral pigeons in sections taken from the same location within HF. Post-hoc tests revealed that homing and Norwich cropper pigeons had significantly higher neuron densities than feral, highflyer, roller, and capuchine pigeons. Feral pigeons had significantly lower neuron densities than homing, show roller, show homer, and Norwich cropper pigeons.

DISCUSSION

Homing pigeons differed marginally from other breeds in the size of HF and its subregions (Hp, APH), but had many more HF neurons and higher neuronal density than most breeds examined. These results suggest that artificial selection for homing behaviour has caused quantitative changes to homing pigeon HF that are primarily observed in neuron number, rather than brain region volume.

One variable that we could not control for was age. HF volume and neuron number vary with age in pigeons (Coppola et al. 2016), so some of the variation within and across breeds could be age-related and not breed-related. Pigeons display no age-related differences in appearance (Burley and Moran 1979), so we had no effective means of aging feral and unbanded domestic pigeons. Of the pigeons for which we had age data ($n = 28$; mean = 2.6 ± 0.995 years, 95% CI), there were significant correlations between age and regional volume (HF, Hp, APH; Pearson's $r = 0.50 – 0.67$, p's ≤ 0.007) and HF neuron number (Pearson's $r = 0.60$, $p < 0.001$), but not HF neuron density (Pearson's $r = 0.35$, $p = 0.07$) (*Appendix 1*), corroborating findings from previous work (Coppola et al. 2016). Despite this, it remains unclear whether previous findings are solely age-related, or due to navigational experience (Cnotka et al. 2008, Coppola et al. 2016, Bingman and Ewry 2020), and we lacked sufficient data to test whether age was a significant covariate across all breeds. Nonetheless, the age range of banded domestic pigeons was quite substantial (1-11 years) which made comparisons to free-living feral pigeons (variant in age) more appropriate. Furthermore, many of the youngest birds (aged 1-2 years) used in this study had similar or higher neuron counts and/or densities compared with some of the oldest birds (aged 8-11 years). Age is therefore unlikely to be a confound in the current study.

However, including it as a covariate with larger sample sizes would allow the effects of age on neuron number and density to be assessed both within and across breeds.

Hp, APH, and HF volumes

Contrary to our prediction, we found little difference in Hp, APH, and HF volumes among domestic pigeon breeds. These results are consistent with Rehkamper et al. (2008) who found that domestic pigeons have larger Hp volumes than wild Rock Pigeons, but found no significant differences among domestic breeds. There are several explanations as to why Hp, APH, or HF volumes do not differ significantly among domestic pigeon breeds. First, homing pigeons are one of the oldest pigeon breeds and have been under several millennia of selection for "homing" (Shapiro and Domyan 2013), a spatial behaviour that integrates sensory cues in order to navigate back to the home loft (Bingman et al. 2003). By contrast, most modern domestic breeds have only recently diverged from ancestral homing pigeons within the last few centuries (Shapiro and Domyan 2013). Second, unlike most other domestic animals, domestic pigeon pedigrees are not well-documented, nor are breeds genetically isolated. Pigeons are often housed in mixed flocks and crossbreeding events are common, both accidentally and for desired traits (e.g., feathered feet, colour patterning). For example, the German Nun pigeon has a mixed heritage of fantail, tumbler, and highflyer pigeons, among others (Biała et al. 2015). Constant breed introgression may result in the maintenance of some neuroanatomical traits across breeds due to genetic linkage. Last, Darwin's theory of evolution by natural selection states that phylogenetic changes are derived from intraspecific variation of the ancestral species (Darwin 1859). Since homing pigeons are the breed by which many domestics descend, most regional brain variation would depend on the variation present in ancestral homing pigeons (Kruska

2005). Thus, there are several reasons why homing pigeons might have similar HF volumes to other domestic breeds.

We also expected homing and feral pigeons to differ in HF volume. While homing pigeons are artificially selected for and practice spatial behaviours, feral pigeons navigate relatively small spatial ranges (Rose et al. 2006) and prioritise energy conservation and hazard avoidance. Due to these differences in behaviour, we expected HF anatomy would differ between domestic and feral pigeons in a similar fashion to trends found between domestic pigeons and wild Rock Pigeons (Rehkamper et al. 2008). However, we found no difference in HF volume between homing and feral pigeons. This lack of difference may be due to the relatively recent introduction of feral pigeons to North America (approx. 400 years) and constant interbreeding with newly escaped domestics. As many as 20% of homing pigeons can be lost during long-distance racing events (Stringham et al. 2012), some of which will join feral pigeon flocks and find mates. Feral pigeons are most genetically similar to homing pigeons, and their relatively short separation from a common ancestor (Stringham et al. 2012) suggests that genetic similarity may be partially responsible for conserved HF volume across populations.

Our findings that HF and Hp volume do not vary based on presumed spatial ability are consistent with several studies of caching (food-storing) bird species. That is, the link between HF size and spatial cognition is not as strong as was initially assumed (Sherry et al. 1989, Sherry et al. 1992). For example, Gould et al. (2013) found no significant differences in Hp volume among corvid species, despite interspecific differences in cache-dependence and spatial task accuracy. Furthermore, a main argument for a correlation between HF/Hp volume and spatial cognition is seasonal size variability: Black-capped Chickadees (*Poecile atricapillus*) have larger HF volumes in autumn when storing food (Smulders et al. 1995) and in harsher environmental

climates where they rely more on caches (Roth and Pravosudov 2009). However, attempts to replicate these findings have been inconclusive (Pravosudov and Roth 2013), and HF volume is smaller in winter when chickadees use spatial knowledge to retrieve cached food items (Bolhuis and Macphail 2001). Pravosudov and Clayton (2002) suggest such differences in HF neuroanatomy are instead caused by differences in working memory capacity which may facilitate, but do not necessary reflect, a bird's ability to navigate in space. Changes in volume may instead represent differences to the number and size of blood vessels, glial cells, and/or neurons, the latter of which may display changes in soma size, dendritic spine density, and/or axon arborisation (Roth et al. 2010). Differences in spatial performance may be better represented by factors that influence the connectivity of HF pathways. Numerous genes regulate the differentiation of HF from other telencephalic regions (Bingman and Ewry 2020), and their variation could vastly influence HF organisation, resulting in differences in spatial cognition. For instance, the *LRP8* gene is a low-density lipoprotein receptor involved in signal induction and long-term potentiation in mammalian Hp (Shao et al. 2020). *LRP8* displays a different pattern of allelic variation in homing pigeon HF compared with other domestics and ferals (Bingman and Ewry 2020, Shao et al. 2020) which could be correlated with variation in spatial cognition. *LRP8* is just one of multiple differentially selected genes in homing pigeons relative to other domestic breeds that influence learning, memory, neurogenesis, and neural organisation in the brain (Shao et al. 2020). Upregulation of one or more of these genes could be responsible for improving spatial cognition in homing pigeons without significantly altering HF volume.

HF neuron number and density

In partial support of our prediction that selection for homing has caused an increase in HF neuron number, homing pigeons have more HF neurons and higher HF neuron densities than

some other domestic breeds (highflyer, roller, capuchine) and feral pigeons. However, homing pigeons display no difference in HF neuron number and density from most show breeds (Norwich cropper, show homer, show roller), despite being better navigators. It is likely that there are multiple factors influencing the number of neurons in HF of pigeons.

Previous studies in birds and mammals indicate that activity level (Uysal et al. 2005, Erickson et al. 2011) and environmental complexity (Day et al. 2008, Freas et al. 2013) positively influence HF volume and HF neuron number, size, and survivorship (Krebs et al. 1989, Cnotka et al. 2008). To account for this, we included birds varying in activity level: show breeds housed in aviaries (least active), free-living feral pigeons that typically perform a few short daily flights to forage (moderately active), and sporting breeds that are allowed multiple hours of daily practice flight (highly active). If exercise and/or environmental complexity are major influencers of HF neuroanatomy, we would expect sporting breeds to have the most HF neurons, followed by feral pigeons, and then show breeds. Homing pigeons had higher absolute neuron counts and densities than capuchine (show), feral, and other sporting pigeons. Moderately active feral pigeons had the lowest HF neuron numbers/densities and several show breeds (Norwich croppers, show rollers, show homers) had higher neuron numbers/densities than non-homing sporting breeds (rollers, highflyers). These results suggest that selection for homing, not general activity level, is at least partially responsible for increased neuron density in the homing pigeon HF.

Interestingly, homing pigeons display no difference in HF neuron number and density from most show breeds (Norwich cropper, show homer, show roller). One potential explanation for the similarity in neuron number between homing pigeons and some of these show breeds is the underlying genetic control of HF neurogenesis and neuron survivorship. As discussed

previously, *LRP8* appears to be a good candidate gene with respect to behavioural and neuroanatomical differences between homing pigeons and other breeds (Bingman and Ewry 2020). Genetic similarities between homing pigeons and show homers could be responsible for similarities in neuron number. Show homers are derived from homing pigeons; most early breeders would assess homing pigeon flocks and keep those of highest quality for the show pen instead of risking them in long-distance racing (Woodfield and Chambers 1892). Instead of being selected for racing performance, show homers were selected for size, shape, and colour. Despite this, show homers are often backcrossed to racing homers, obstructing the potential for genetic differences that might impact HF neuroanatomy. Furthermore, most show homers will still home if trained properly (Woodfield and Chambers 1892), suggesting constant breed introgression has upheld the behaviour. Unlike dog breeds, in which strict pedigrees are maintained, show pigeons are often the product of crossing many different breeds to introduce desirable and/or novel traits. For example, the American show roller is a relatively recent breed often backcrossed to other breeds for specific morphological traits. It is therefore possible that many of the show pigeons we sampled were recently derived from and/or crossed with homing and other pigeons, leading to neuroanatomical similarities across breeds.

Another potential reason for a lack of differences in HF neuron number and density between homing pigeons and domestic show breeds could be selection for other behaviours. Norwich croppers had comparable HF neuron numbers to homing pigeons and slightly higher neuron densities than homing pigeons (Table 2.3). Cropper pigeons have been an outlier in previous studies (Rehkamper et al. 2008), which is not uncommon for domestic breeds under selection for extreme characters (Frahm and Rehkamper 1998). Cropper pigeons were originally bred to steal female pigeons during thieving competitions or directly from competitors' lofts

(Hiatt and Esposito 2000). As such, croppers possess exaggerated secondary sexual characters that make them desirable to mates, e.g., extremely upright body posture, enlarged crop, and the tendency to perform sexual displays almost constantly (*Figure 2.1B*) (Hiatt and Esposito 2000). Sexual displays in birds are largely modulated by sex hormones (Fusani 2008) and androgen levels influence HF morphology and function. For example, testosterone can improve HF cognitive function, affect neuronal morphology, and increase the survival of HF neurons (Atwi et al. 2016). If cropper pigeons are selected for constant "hyper-sexualization" (Hiatt and Esposito 2000), then increases in testosterone levels in displaying males might influence HF neuron survival (Atwi et al. 2016). While the relationship between androgens and HF are not well documented with regards to the spatial behaviour of avian species, studies of songbirds demonstrate sex hormones significantly contribute to long-term memory and learning plasticity, and the growth of neural circuitry within the avian brain (Schlinger and Saldanha 2005) during periods of high courtship (Metzdorf et al. 1999). Similarities in HF neuron number and density between croppers and homers could therefore be explained by androgen-related effects on neurogenesis (Atwi et al. 2016) caused by selection for sexual traits (Metzdorf et al. 1999, Schlinger and Saldanha 2005) independent of spatio-cognitive performance. Unfortunately, we still lack data on whether cropper pigeons display measurable differences in androgen hormone levels from other pigeon breeds.

We also predicted that the stress of wild living might result in a decrease in neuron number in feral pigeons compared to domestic breeds. Chronic stress reduces survival of new neurons in the avian HF (Robertson et al. 2017, Smulders 2017, Gualtieri et al. 2019) and could be a contributing factor to observed reductions in Hp neurogenesis (Sherry and MacDougall-Shackleton 2015). Furthermore, differences in resource availability could limit the amount of

metabolic energy available for the development and maintenance of neuron populations (Lesch et al. 2022). Domestic pigeons have shelter and food provided *ad libitum*, whereas feral pigeons have less consistent food and water sources and are routinely presented with threats both living and non-living (predators, vehicles, traps/poisons). In accordance with this prediction, feral pigeons have significantly less HF neurons and lower neuron densities than homing, show homer, show roller, and Norwich cropper pigeons. Notably, ferals have less than half the number of neurons (*Figure 2.5A*) and less than half the neuronal density (*Figure 2.5C*) of homing pigeons. This more than 2-fold decrease in neuron number and density is especially interesting given homing and feral pigeons are more genetically similar to each other than to other breeds (Stringham et al. 2012). Feral pigeons are made up of escaped homing (and occasionally other domestic) pigeons no longer under artificial selection for homing performance, and navigate relatively small spatial ranges (Rose et al. 2006) to prioritise energy conservation and hazard avoidance. A lower reliance on navigation in a small spatial range could result in a reduction to HF neuron number, but feral pigeons display fewer neurons even than inactive show breeds, suggesting stress might also contribute to reduced neuron density in feral pigeon HF. If stress negatively impacts HF neuron number, then feral pigeons might also display a reduced capacity for learning in comparison to domestic pigeon breeds that experience less stress over their lifetime.

Conclusions

Homing is an innate behaviour (Gagliardo et al. 2007) wherein practiced components of spatial cognition are used to return to a home loft by a most direct route (Whishaw et al. 2001). Homing pigeons are better navigators than feral and show breeds (Woodfield and Chambers 1892, Edrich and Keeton 1977) and based on our data, this is at least partially associated with

higher neuron density in HF, the brain region that controls spatial behaviour (Bingman et al. 2005, Sherry 2006). This is the first evidence of artificial selection for a cognitive task driving a regional change in neuron number and density. Furthermore, these results are novel in that regional neuronal density is not accompanied by a concomitant change in brain region volume. Future directions should study the genes correlated with increased HF neuron number and/or the extent to which HF neuron survivorship depends on lifetime homing experience, factors that might significantly influence HF morphology (Cnotka et al. 2008).

Table 2.1. Descriptive statistics for body and brain mass measurements (in g) across eight pigeon breeds (n = 51). All values are reported by breed average (mean) and standard deviation (SD).

Breed	n	Body mass (g)		Brain mass (g)	
		Mean	SD	Mean	SD
Feral	8	407	50.7	2.23	0.19
Homer	10	522	33.6	2.28	0.11
Highflyer	7	415	25.3	2.06	0.14
Roller	6	421	43.9	2.07	0.16
Capuchine	4	499	35.2	2.22	0.28
Show roller	6	433	24.9	2.03	0.12
Show homer	5	569	40.4	2.23	0.14
Cropper	5	516	56.7	2.50	0.13

Table 2.2. Descriptive statistics for four measurements of brain anatomy across eight pigeon breeds (n = 51). Measurements include telencephalon, hippocampus, area parahippocampalis, and hippocampal formation volumes (in mm^3). All values are reported by breed average (mean) and standard deviation (SD).

Breed	n	Telencephalon volume (mm^3)		Hippocampus volume (mm^3)		Area parahippocampalis volume (mm^3)		Hippocampal formation volume (mm^3)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Feral	8	977.1	158.0	33.6	5.2	24.1	3.1	57.7	5.8
Homer	10	992.1	136.5	38.7	6.2	25.4	6.6	64.2	8.5
Highflyer	7	923.5	62.3	30.8	2.7	20.3	5.7	51.0	7.3
Roller	6	907.2	71.9	30.4	3.7	26.9	3.9	57.3	3.4
Capuchine	4	960.9	114.8	37.3	4.6	20.2	4.5	57.3	4.8
Show roller	6	880.5	87.1	34.5	5.0	20.7	3.0	55.2	7.5
Show homer	5	928.3	75.3	35.2	2.1	21.5	1.5	56.7	1.9
Cropper	5	1166.3	69.5	38.2	5.7	23.0	1.2	61.3	6.2

Table 2.3. Descriptive statistics for two measurements of hippocampal formation (HF) anatomy across eight pigeon breeds (n = 51): neuron number and neuron density (in n/mm^3). All values are reported by breed average (mean) and standard deviation (SD).

Breed	n	HF neuron number		HF neuron density (/mm^3)	
		Mean	SD	Mean	SD
Feral	8	1072185	255997	18708	4657
Homer	10	2426353	593592	37601	6925
Highflyer	7	1276222	245974	25288	5162
Roller	6	1348679	193011	23538	2970
Capuchine	4	1328841	587279	22720	8483
Show roller	6	1851884	333582	33787	6117
Show homer	5	1853808	172313	32745	3532
Cropper	5	2428458	442159	39980	8859

Table 2.4. Results of a Fisher's one-way analysis of variance (ANOVA) comparing absolute measurements across eight pigeon breeds (n = 51). Data were compared by body and brain mass (in g); volumetric differences (in mm^3) in telencephalon, hippocampal formation (HF), hippocampus, and area parahippocampalis size; and HF neuron number and density (in n/mm^3). ANOVA results are reported using F-ratios (F), degrees of freedom (df), and p-values, wherein (*) denotes a significant difference among breeds. Tukey's honestly significant difference (HSD) post-hoc tests reveal pairwise differences. Relationships relative to the homing pigeon breed are represented using the greater than (>) symbol and non-significance is represented by (NS).

Measurements	F	df	p-value	Tukey HSD
Body mass	15.02	7, 43	**<0.001***	homer > feral, highflyer, roller, show roller
Brain mass	5.55	7, 43	**<0.001***	NS
Telencephalon volume	3.52	7, 43	**0.004***	NS
Hippocampus volume	2.99	7, 43	**0.012***	homer > highflyer, roller
Area parahippocampalis volume	2.07	7, 43	0.068	NS
Hippocampal formation volume	2.87	7, 43	**0.015***	homer > highflyer
HF neuron number	12.77	7, 43	**<0.001***	homer > feral, highflyer, roller, capuchine
HF neuron density	11.15	7, 43	**<0.001***	homer > feral, highflyer, roller, capuchine

Table 2.5. Results of analyses of covariance (ANCOVAs) comparing relative log-transformed measurements of neuroanatomy by breed, covariate, and overall model effects across eight pigeon breeds (n = 51). Telencephalon volume (in mm^3) was compared using total brain volume (minus CB, telencephalon) as a covariate; volumetric differences in hippocampal formation (HF), hippocampus (Hp), and area parahippocampalis (APH) size used telencephalon volume (minus region of interest) as a covariate; HF neuron number used HF volume as a covariate. ANCOVA results are reported using F-ratios (F), degrees of freedom (df), and p-values, wherein (*) denotes a significant difference among breeds. Tukey's HSD post-hoc tests reveal pairwise differences. Relationships relative to the homing pigeon breed are represented using the greater than (>) symbol and non-significance is represented by (NS).

Measurements	Breed effects				Covariate effects				Model		
	F	df	p-value	Tukey HSD	Covariate	F	df	p-value	F	df	p-value
Brain mass	5.04	7, 42	**<0.001***	NS	Body mass	20.12	1, 42	**<0.001***	8.94	8, 42	**<0.001***
Telencephalon volume	2.61	7, 42	**0.025***	NS	Brain volume – Tel, CB	2.12	1, 42	0.153	2.96	8, 42	**0.01***
Hippocampus volume	2.37	7, 42	**0.039***	NS	Tel – Hp volume	8.05	1, 42	**0.007***	3.96	8, 42	**0.001***
Area parahippocampalis volume	2.37	7, 42	**0.039***	NS	Tel – APH volume	3.56	1, 42	0.066	2.56	8, 42	**0.023***
Hippocampal formation volume	2.73	7, 42	**0.020***	homer > highflyer	Tel – HF volume	12.88	1, 42	**<0.001***	4.88	8, 42	**<0.001***
HF neuron number	10.94	7, 42	**<0.001***	homer > feral, highflyer, roller, capuchine	HF volume	8.26	1, 42	**0.006***	14.10	8, 42	**<0.001***

Figure 2.1. Comparison of several breeds of pigeon: A. feral pigeon, descendant of escaped domestic pigeons; B. Norwich cropper, a show breed selected for secondary sexual characters; C. capuchine, a show breed selected for head crest feathers; D. roller, a sporting breed selected for in-flight somersault (rolling) behaviour; and E. racing homer, a sporting breed selected for spatial navigation. Image of homing pigeon courtesy of Francisco Seco (x); image of feral pigeon courtesy of ebird.org (x).

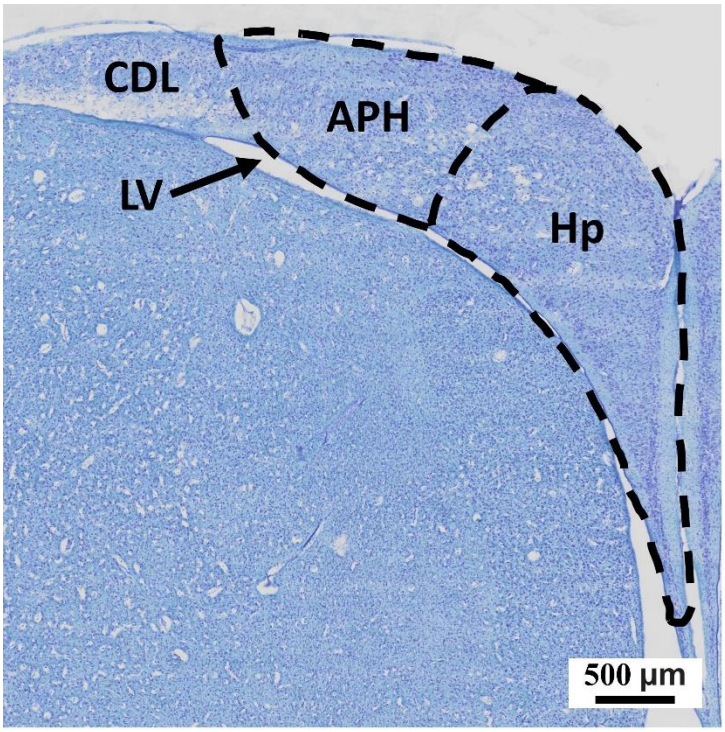

Figure 2.2. Border outlines for hippocampus (Hp) and area parahippocampalis (APH) in a mid-section of a single telencephalic hemisphere of a homing pigeon stained for Nissl bodies using thionin acetate. Hp is bordered by a dense layer of cells, and APH continues until separated by an upward indentation of the lateral ventricle (LV), just before the dorsolateral corticoid area (CDL). Together, the Hp and APH make up the hippocampal formation (HF).

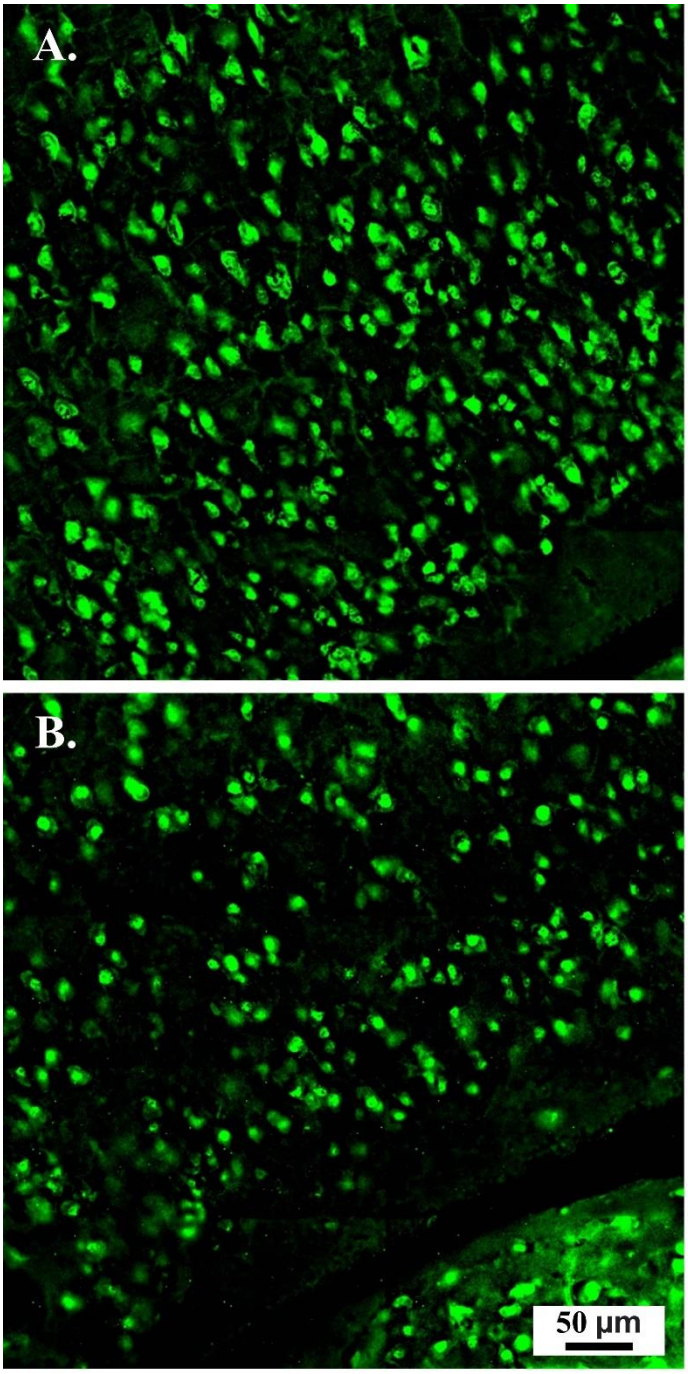

Figure 2.3. Hippocampal formation neurons immunolabeled for the neuron specific antigen NeuN from the same medial location along the lateral ventricle in A. homing and B. feral pigeons. For reference, the nidopallium and lateral ventricle are partially visible in the bottom right corner.

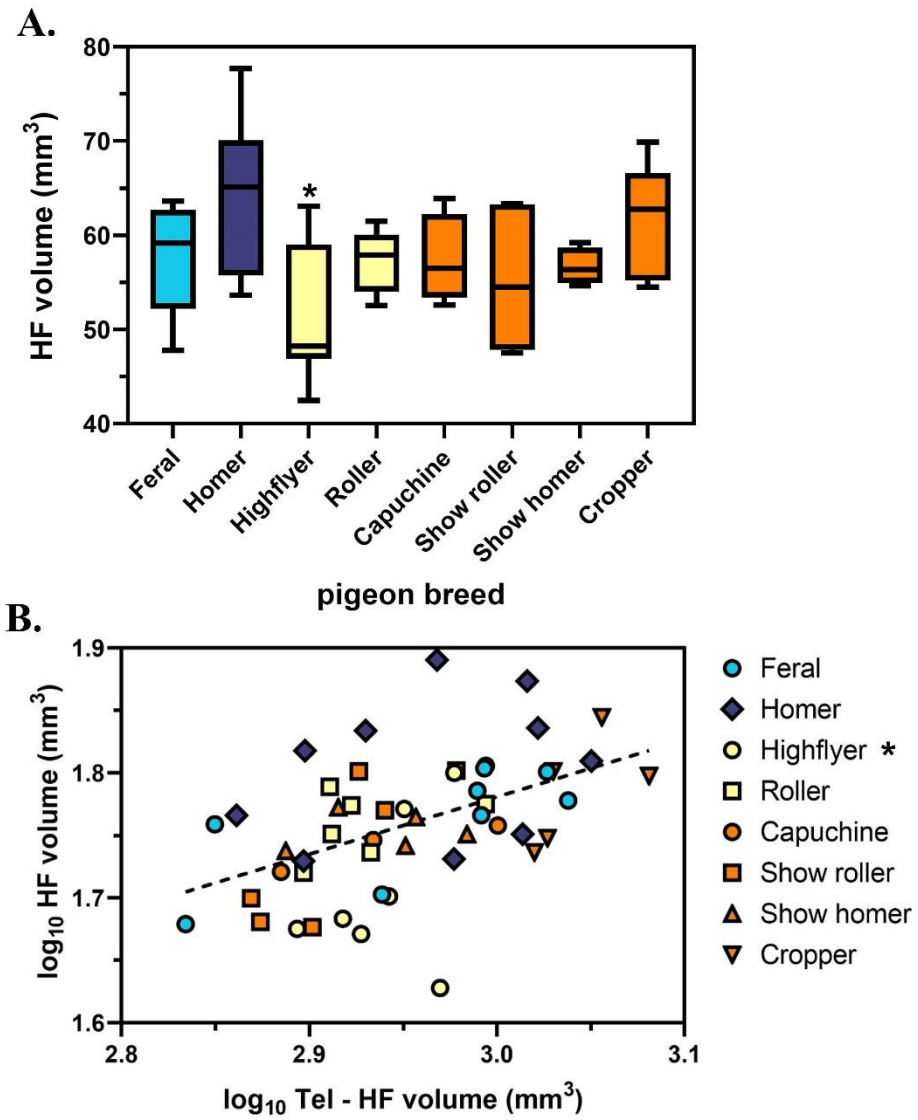

Figure 2.4. Among-breed differences in A. absolute HF volume (mm^3) with min and max range and B. log-transformed HF volume relative to log-transformed telencephalon (minus ROI) volume. As per the legend, ferals = light blue, homers = dark blue, sporting breeds = yellow, show breeds = orange. The dotted line represents the allometric relationship (least-squares linear regression) between x and y across all breeds, and (*) denotes a significant difference from homing pigeons ($p < 0.05$).

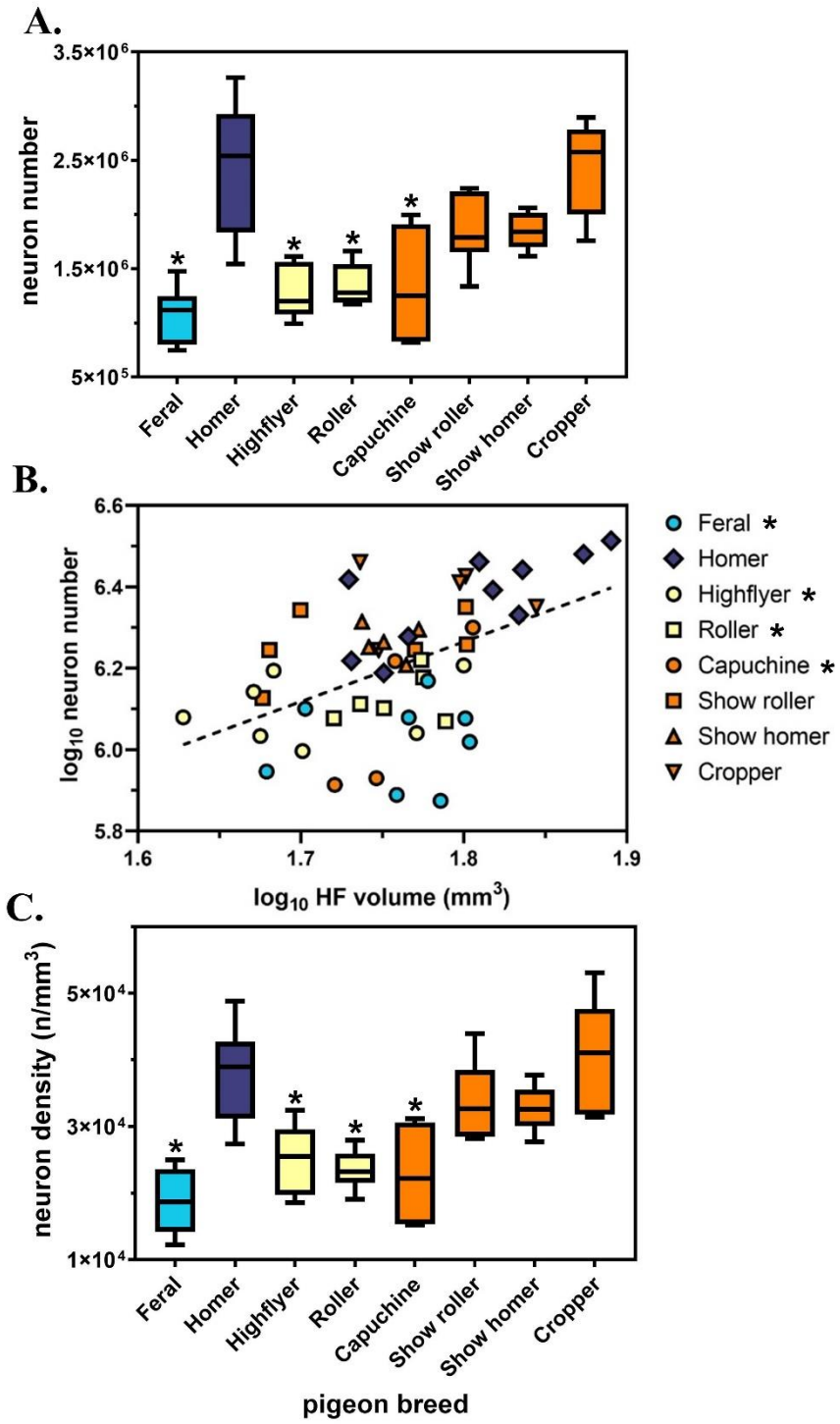

Figure 2.5. Among-breed differences in A. absolute HF neuron number with min and max range; B. log-transformed HF neuron number relative to log-transformed HF volume; and C. absolute neuron density (n/mm^3) with min and max range. As per the legend, ferals = light blue, homers = dark blue, sporting breeds = yellow, show breeds = orange. The dotted line represents the allometric relationship (least-squares linear regression) between x and y across all breeds, and (*) denotes a significant difference from homing pigeons ($p < 0.05$).

CHAPTER THREE: QUANTITATIVE DIFFERENCES IN THE NEUROANATOMY OF SEPTUM ACROSS DOMESTIC PIGEON (*COLUMBA LIVIA*) BREEDS

INTRODUCTION

The process of domestication involves selectively breeding plants and animals for specific traits (Kruska 1988, Francis 2015). Captive domestic animals are no longer influenced by natural selective pressures and are instead subject to human-influenced "artificial" selection. In all cases, domestic animals are selected for ease of handling, or tameness (Kruska 1988). Tame animals exhibit lower emotional responsiveness and higher tolerance to manmade stressors, such as high density herd-living and frequent interactions with humans (Price 1984). Despite selection for tameness across species, artificial selection has also driven interspecies and interbreed differences in both behaviour and morphology. Depending on intended purpose, domesticated species have undergone selection for performance (e.g., speed, strength), production (e.g., meat, fabrics), and/or morphology (e.g., conformation, colour pattern) (Kruska 1988). The extensive variation between breeds of domestic animals also extends to the brain. For example, domestic breeds have relatively smaller brains than their wild ancestors (Kruska 1988, Agnvall et al. 2017). This 'regression' is likely due to a loss of complex survival behaviours no longer necessary for tame animals that rely on humans for food, shelter, and protection (Agnvall et al. 2017). Alongside whole brain differences, altering behavioural complexity in domestic species may also affect the relative and absolute size of brain regions or systems (Kruska 1988).

Although much of the brain is influenced by artificial selection processes, the limbic system is of particular interest because it regulates complex emotional and behavioural responses related to survival, such as feeding, reproducing, and fight-or-flight behaviours (Kolb et al. 2001). The limbic system is comprised of a number of regions, including the hippocampal formation (HF), hypothalamus, amygdala, and septum (Szekely 1999). HF is involved in spatial

navigation (Pravosudov and Clayton 2002, Jacobs 2003, Bingman et al. 2005, Gould et al. 2013) and emotionally-guided behaviour (Kruska 1988), the hypothalamus produces hormones for regulating survival behaviours (e.g., hunger, thirst) (Smulders 2017), the amygdala produces fight-or-flight responses to threatening stimuli (Kolb et al. 2001), and the septum is involved in both agonistic and copulatory conspecific signalling (Ramirez et al. 1988, Corrales Parada et al. 2021). In comparison to wild-type ancestral species, the limbic pathway of domestic species has been altered significantly (Ebinger 1974, Kruska 1988, Kruska 2005). HF has decreased most of any brain region (41- 44%) (Ebinger 1974, Kruska 2005), followed by amygdala (25.5%), and septum (17.4%) (Ebinger 1974). Overall, domestic birds and mammals have decreased the total volume of telencephalic limbic structures by as much as 40% (Ebinger 1974, Kruska 1988, Ebinger 1995, Kruska 2005). Decreases in limbic region size are often attributed to selection for tameness and living in a captive environment at higher densities than would be typical of the wild type (Kruska 2005, Agnvall et al. 2017).

In birds, septum is involved in a wide range of behavioural functions including social communication, pair-bonding, sexual behaviours, and aggression (Ramirez et al. 1988, Goodson et al. 2005, Corrales Parada et al. 2021). Specifically, lateral septum (LS) (Ramirez et al. 1988) plays a critical role in modulating aggression (Brady and Nauta 1953, Lubar and Numan 1973, Kruska 2005). LS lesions reduce defensive and increase offensive behaviours toward conspecifics (Ramirez et al. 1988, Goodson et al. 1998) and immediate early gene (IEG) expression increases in septal regions when a novel, and potentially threatening, conspecific partner is introduced (Corrales Parada et al. 2021). Lesion, electrical stimulation, and hormone manipulation studies on septum all affect agonistic communication and sociality (Goodson et al. 2005). Additionally, septum appears to have a role in sexual behaviour, particularly in males

(Viglietti-Panzica et al. 1992, Taziaux et al. 2006). The importance of septum in avian behavioural modulation is likely due to its high connectivity with several other structures involved in social decision-making. These include direct connections with HF (Atoji and Wild 2006), hypothalamus (Goodson et al. 2004, Reiner et al. 2004), and the extended medial amygdala (Goodson et al. 2004, Montagnese et al. 2004). Due to its connectivity with limbic structures, in particular HF, it is highly likely that septum plays a role in goal-oriented spatial behaviour (Peterson and Bingman 2011), and/or in modulating aggression or sexual behaviours (Montagnese et al. 2004), all of which have been altered in domestics compared to their wild ancestors (Price 1984).

The domestic pigeon (*Columba livia*; *Figure 3.1A-E*) is an ideal species in which to test for breed differences in septum anatomy because pigeon breeds exhibit more behavioural trait variation than any other domesticated bird species (Price 2002). For example, homing pigeons (*Figure 3.1E*) have been selected for nearly 5,000 years for their 'homing' ability (Shapiro and Domyan 2013). While wild Rock Pigeons can locate a cliffside nest up to 80 kilometres away (Alleva et al. 1975), homing pigeons can return home from an unfamiliar location as far as 1,000 kilometres away, making them better navigators than both wild Rock Pigeons and other domestic breeds (Edrich and Keeton 1977, Degner and Blechman 2011). Homing is not the only artificially selected behaviour in pigeons: roller pigeons (*Figure 3.1D*) are selected to perform short tumble sequences through the air (Mowrer 1940), whereas Norwich cropper pigeons (*Figure 3.1B*) are selected for exaggerated secondary sex characters that make them highly desirable to potential mates. Croppers display their enlarged crop (among other features) almost constantly, and were originally used to steal mates during 'thieving' competitions and from competitors' lofts (Darwin 1894, Hiatt and Esposito 2000). Additionally, like all domestic

species, domestic pigeons have been selected for tameness and are therefore more docile than wild-living feral pigeons (Daniels and Bekoff 1989) that have been released from frequent close-contact with humans and artificial selective pressures.

The septum has roles in spatial working memory (Peterson and Bingman 2011), sexual behaviour (Viglietti-Panzica et al. 1992), and aggression (Goodson et al. 2005), all of which vary across pigeon breeds. Despite this variation, previous studies found no differences in septum size across breeds (Rehkamper et al. 1988, Frahm and Rehkamper 1998), but these studies focused only on septum volume when neuron number and/or density may be more closely associated with behavioural differences (Roth et al. 2010, Herculano-Houzel 2018). Here, we quantify septum anatomy across pigeon breeds to determine if differential selection for behaviour has caused changes to septum size, neuron number, and/or neuronal density. Based on the roles that the septum has in modulating behaviour, we made several possible predictions. First, homing pigeons would have a larger septum with more neurons than most show breeds and other sporting breeds because of the presumed role of septum in some aspects of spatial memory (Peterson and Bingman 2011). Second, feral pigeons would differ in septum size and neuron numbers from tamer, less agonistic domestic pigeons. Finally, Norwich croppers, selected for enhanced sexual characters, would have larger septum volumes and/or more neurons than other domestic pigeon breeds.

MATERIALS AND METHODS

Animals

A total of 39 male and female domestic pigeons (*Columba livia*) were used, including feral pigeons (n = 7), homing pigeons (n = 8), sporting breeds (rollers, n = 4; highflyers, n = 5), and show breeds (Norwich croppers, n = 6; show homers, n = 5; American show rollers, n = 4).

All homing pigeons were trained and used in local races. All other breeds were sourced from pigeon breeders across Alberta and Saskatchewan. Feral pigeons were trapped using standard wire baited traps in Lethbridge and Hannah, Alberta. Birds were immediately weighed (Table 3.1) and sacrificed using an intracoelomic injection of sodium pentobarbital (2 mL/kg body weight) before being perfused transcardially with 0.1 M phosphate buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde (PFA). The brains and eyes were removed and stored in 4% PFA at 4 ^{o}C for 1 to 2 weeks. Brains were weighed (Table 3.1), cryoprotected in 30% sucrose PBS solution, moved into antifreeze, and stored at -20 ^{o}C until embedding. All animals were handled according to Canadian Council on Animal Care (CCAC) guidelines and animal welfare policies at the University of Lethbridge (Animal Welfare Protocol #2011).

Histology

Before histological processing, the cerebella were first dissected and stored for future projects. Brains were then embedded in gelatin blocks and sectioned coronally on a freezing stage microtome at 40 µm thickness. All sections were collected in PBS + 1% sodium azide solution in multi-well plates. For all specimens, every fourth section (1:4 series) was mounted onto gelatinised slides. Once dry, sections were bathed in chloroform and stained for Nissl bodies using thionin acetate followed by a graded ethanol series. Sections were cleared in Hemo-De (Thermo Fisher Scientific, HD150A) and coverslipped with Permount (Thermo Fisher Scientific, #SP15500).

Five to 10 sections throughout the rostrocaudal extent of septum were immunolabeled for the neuron specific antigen, NeuN (Cao et al. 2002). Sections were rinsed in 1% PBS (pH 7.4), then incubated in 10% normal goat serum (Jackson ImmunoResearch, 005-000-121) at a 1:10 dilution in 1% PBS + 0.025M Triton (PBST) for 1 hour. The sections were then incubated in a

monoclonal mouse anti-NeuN primary antibody (clone A60, Sigma Aldrich, MAB377) at a 1:1000 dilution in 0.0125M PBST on a shaker plate for 24 hours at room temperature. The sections were rinsed again in PBS and then incubated for 4 hours in fluorescein (FITC) goat anti-mouse secondary antibody (Jackson ImmunoResearch, 111-095-144) at a 1:200 dilution in 0.0125M PBST on a shaker plate at room temperature. Finally, the sections were rinsed and mounted onto gelatinised slides.

Stereology

All volumetric measurements and neuron counts were done using unbiased stereology (Howard and Reed 2004). Regions of interest (telencephalon and septum) were differentiated according to multiple stereotaxic atlases of bird brains (Karten and Hodos 1967, den Boer-Visser et al. 2004, Puelles et al. 2018) (*Figure 3.2A*). Volumetric measurements were made on a Zeiss Axio Imager M2 microscope using the Cavalieri Estimator Probe in StereoInvestigator^{TM} with a 400 µm grid size for all regions of interest. Quantifications for total brain and telencephalon were taken using a 1x objective lens and septum (*Figure 3.2A*) was quantified using a 2.5x objective lens due to its smaller size (Table 3.1). Coefficients of error (Gunderson, m = 1) for all volumes were < 0.055 with a 95% confidence interval (CI) < 0.06 for all specimens.

The total number of neurons within septum were estimated using the Optical Fractionator method (Altunkaynak et al. 2011), as implemented in StereoInvestigator^{TM}. The following parameters were used: a grid spacing of 350 µm, a grid size of 40 µm, a dissector zone of 15 µm, and upper and lower guard zones of 5 µm. Neuron counts were completed using a 40x immersion oil lens on a Zeiss Axio Imager M2 microscope. Neurons labelled with NeuN (*Figure 3.3A-D*) were counted if at least two-thirds of the cell membrane was visible and at least two-

thirds of the neuron came into focus within the dissector (Table 3.2). Coefficients of error (Gunderson, m = 1) for all cell counts were < 0.10 with a 95% CI < 0.15 for all specimens.

Statistical Analyses

All statistical analyses were performed in Jamovi, an open-source statistics software built using R statistical language (The jamovi project 2022). Only birds from which both volume and neuron counts were measured were included in the analyses (n = 39). Absolute values were analysed using Fisher's one-way analysis of variance (ANOVA) and a Tukey's honestly significant difference (HSD) post-hoc test. Relative values were analysed using analyses of covariance (ANCOVAs) of log-transformed data, with pairwise comparisons made with Tukey's HSD post-hoc tests. ANCOVAs used telencephalon volume (minus region of interest (ROI)) or septum volume as covariates. Interaction effects (breed x covariate) were not significant for all variables, indicating no significant differences in slope among breeds. Interaction effects were therefore removed from final ANCOVA models.

RESULTS

Septum volumes

Norwich croppers had the largest septum volumes (mean = 14.13 mm^3), whereas rollers (mean = 10.3 mm^3), homers (mean = 10.4 mm^3), and highflyers (mean = 10.5 mm^3) had the smallest septum volumes (Table 3.1). Analyses of absolute data showed septum volume differed significantly among breeds (Table 3.3). A Tukey's HSD post-hoc test revealed Norwich croppers had significantly larger septum volumes than feral, homer, highflyer, and roller pigeons (*Figure 3.4A*), but there were no significant differences among any other breeds.

Relative septum volume also differed significantly among breeds (Table 3.4). Post-hoc tests revealed homers had significantly smaller septum volumes than show roller, show homer, and Norwich cropper pigeons. This can also be observed in the scatterplot shown in *Figure 3.4B*; show breeds tend to have larger relative septum volumes than the population average, whereas homing pigeons tend to be below the regression line depicting the allometric relationship between septum and telencephalon volumes across all breeds.

Septum neuron number and density

On average, Norwich cropper pigeons had the most neurons (mean = 5.8×10^5), and feral pigeons had the fewest neurons (mean = 2.0×10^5) (Table 3.2). The absolute number of septum neurons differed significantly among breeds (Table 3.3). Post-hoc tests revealed that Norwich croppers had significantly more septal neurons than feral, homer, and roller pigeons, whereas feral pigeons had significantly fewer neurons than highflyer, show roller, show homer, and Norwich cropper pigeons (*Figure 3.5A*). There were no significant differences among other breeds.

Septum neuron number relative to septum volume also differed significantly among breeds (Table 3.4). A Tukey's HSD post-hoc test revealed feral pigeons had significantly fewer relative septum neurons than highflyer, show roller, show homer, and Norwich cropper pigeons. Similarly, roller pigeons had significantly fewer septum neurons than highflyer and Norwich cropper pigeons. *Figure 3.5B* shows that most sporting and show breeds trend along or above the whole-population average, whereas roller and feral pigeons trend below the whole-population average. Septum volume did not have a significant influence on septum neuron number, but the overall ANCOVA model was still significant.

Septum neuron density, calculated by dividing neuron number by septum volume, also differed significantly among breeds (Table 3.3). Highflyer and Norwich cropper pigeons had the highest septum neuron densities (mean = 4.5×10^4 neurons/mm^3 and mean = 4.1×10^4 neurons/mm^3, respectively), and feral and roller pigeons had the lowest septum neuron densities (mean = 1.9×10^4 neurons/mm^3 and mean = 2.2×10^4 neurons/mm^3, respectively) (Table 3.2). *Figure 3.3* shows differences in neuronal density among homing, feral, highflyer, and Norwich cropper pigeons. Post-hoc tests revealed that highflyers had significantly higher neuron densities than feral and roller pigeons, whereas feral pigeons had significantly lower neuron densities than highflyer and Norwich cropper pigeons (*Figure 3.5C*).

DISCUSSION

Norwich cropper pigeons, selected for exaggerated sexual characteristics, have significantly larger absolute septum volumes and septum neuron counts than most domestic breeds and feral pigeons. In contrast to croppers, homing pigeons had significantly smaller relative septum volumes, and demonstrated no difference in neuron number and density from other breeds. These results suggest that artificial selection for sexual characters, but not spatial working memory, may partially influence septum volume.

Variables we could not control for included both age and sex, which are indistinguishable in adult pigeons (Burley and Moran 1979). We were most interested in sex effects, as males and females can display regional sexual dimorphism in neuron number and morphology (Viglietti-Panzica et al. 1992, Taziaux et al. 2006). Most specimens were sexed during dissections post-perfusion (n = 30), and the sex ratio was relatively consistent (males = 14; females = 16). Independent sample t-tests comparing male and female pigeons found no differences in septum volume, neuron number, or neuron density between sexes ($t = 0.159 – 0.978$; $p \geq 0.336$)

(*Appendix 2*). Therefore, it is unlikely that sex had a significant influence on our results. Next, we looked at age-related effects on septum neuroanatomy since age can influence regional volume and/or neuron number in bird species (Coppola et al. 2016). Similarly, older pigeons have substantially fewer septal choline acetyltransferase (ChAT)-expressing cells compared to younger pigeons, which could be in part responsible for reduced spatial cognition in older pigeons (Coppola et al. 2021). Of the pigeons for which we had age data (n = 23; mean = 2.8 ± 1.136 years, 95% CI), there were no significant correlations between age and septum volume, neuron number, or neuron density (Pearson's r = -0.384 to -0.048, p = 0.070 to 0.827) (*Appendix 3*). While age may contribute to declines in ChAT-expressing cells (Coppola et al. 2021), it is unlikely that age significantly influenced neuronal differences in this study.

Septum and HF are highly interconnected (Goodson et al. 2004, Coppola et al. 2021), and septum lesions impair spatial working memory in pigeons (Peterson and Bingman 2011). We had therefore predicted homing pigeons would have larger septum volumes with more neurons than other breeds. Contrary to our prediction, homing pigeons had smaller septal volumes and fewer neurons than show breeds. These results suggest a lack of relationship between spatial cognition performance and septum neuroanatomy. Goodson et al. (2004) proposed that medial septal neurons may be involved in aspects of learning and memory (Shiflett et al. 2002, Goodson et al. 2004) which may facilitate spatial navigation, but do not directly influence spatio-cognitive performance traits. Thus, the septum may be insufficiently involved in homing to cause significant differences in volume or neuron number in homing pigeons compared to other pigeon breeds.

Apart from selection for homing ability, all domestic pigeons have been selected for thousands of years for "tameness". Tame animals exhibit higher tolerance to biological and/or

physical stressors, limiting the negative effects of captivity, e.g., high density herd-living, frequent human contact (Price 1984). As such, domestic pigeons are more docile than wild-living feral pigeons (Daniels and Bekoff 1989) that have been released from artificial selective pressures for tameness traits. Septum is involved in modulating aggression and fearfulness (Montagnese et al. 2004), both of which are altered in domestic animals. Lesion, electrical stimulation, and hormone manipulation studies show that the septum influences agonistic communication and sociality (Goodson et al. 2005). Specifically, LS lesions increase aggression toward conspecifics in both birds and mammals (Brady and Nauta 1953, Lubar and Numan 1973, Ramirez et al. 1988, Goodson et al. 1998, Kruska 2005). Due to differences in selection for tameness, we predicted that feral pigeons might have an enlarged septum with more neurons than more docile domestic breeds. Contrary to our prediction, feral pigeons had fewer neurons and lower neuronal density in the septum compared with most domestic pigeon breeds. This finding could be a consequence of life history since feral pigeons are subject to higher ambient environmental stress, including predation risk and food scarcity. The impact of stress on neuroanatomy has been primarily investigated in HF, wherein chronic prolonged stress reduces new neuron survivorship (Robertson et al. 2017, Smulders 2017, Gualtieri et al. 2019), potentially contributing to reduced neuron density. The septum is certainly activated in response to social stressors (Goodson et al. 2005, Muigg et al. 2007), and rats increase IEG expression in septum in response to stressful stimuli, suggesting stress susceptibility at the neuronal level (Muigg et al. 2007). Stress could be a contributing factor to observed reductions in hippocampal neurogenesis in captured wild birds (Sherry and MacDougall-Shackleton 2015), and septum may be similarly affected. However, more research is needed to understand the full influence of stress on the quantitative neuroanatomy of limbic structures.

Last, we predicted the role of septum in regulation of sex-related behaviours might cause increased septum volume or neuron number in breeds selected for exaggerated sexual characters. The septum plays a significant role in modulating agonistic and courtship behaviours (Viglietti-Panzica et al. 1992, Goodson et al. 1998, Taziaux et al. 2006). In pigeons, LS lesions cause increased offensive behaviours toward conspecifics (Ramirez et al. 1988), and septal lesions simplify or reduce both agonistic and courtship behaviours in male songbirds (Goodson et al. 1998). We specifically predicted that Norwich croppers would have an enlarged septum with more neurons than other domestic breeds. Cropper pigeons are likely derived from the Horseman Thief Pouter, a breed selected since the 17th century to compete against conspecifics for mates (Keefe 2017). Croppers use exaggerated sexual characters and displays to steal female pigeons, either during organised thieving competitions or directly from competitors' lofts (Hiatt and Esposito 2000). During organised events, as many as 50 males are released in the presence of a single female. Males must be able to distinguish the female in a large flock, outlast other males, and finally the most enticing male may return her to his loft to be declared the winner (Hiatt and Esposito 2000). Sexual display behaviours contain both agonistic (e.g., chasing, bowing) and courtship (e.g., nest-soliciting) elements (Fusani 2008), both of which are likely controlled by septum (Goodson et al. 1998). The intense selection for courtship in croppers could therefore drive an increase in septum size. In accordance with our prediction, Norwich cropper pigeons have larger absolute septum volumes than feral, homing, highflyer, and roller pigeons and higher absolute neuron counts than feral, homing, and roller pigeons (Table 3.3). Cropper neuron density differed only from feral pigeons, meaning that their enlarged septum likely does not arise from a disproportionate number of neurons.

Behavioural responses are controlled by the limbic system, which involves the amygdala, hypothalamus, and hippocampal formation, all of which are highly connected to septum (Atoji and Wild 2006). The central placement of septum suggests its function in a variety of different behavioural contexts, e.g., arousal, anxiety, defense (Goodson et al. 1998, Goodson et al. 2004). Broadly, septum appears to be involved in integration of various social and stress responses (Lubar and Numan 1973, Corrales Parada et al. 2021). Within avian septum, Goodson et al. (2004) identify two main regions: LS and medial septum (MS). LS and MS control different aspects of limbic system functioning (Ramirez et al. 1988, Goodson et al. 2004), but are not reliably discernible in the Nissl- or NeuN-stained tissue used in this study. MS activates when presented with same-sex conspecific stimuli, suggesting a possible role in sexual behaviours (Goodson et al. 2005). LS contains both steroid hormone receptors and testosterone-sensitive arginine vasotocin-immunoreactive fibers, both of which are involved in regulating social communication and further suggest the septum's involvement in a variety of agonistic and social behaviours (Goodson et al. 2004). Since sexual display behaviours contain both agonistic and courtship elements (Fusani 2008), it is possible that breeds such as the Norwich cropper, selected for exaggerated sexual characters, display higher neuron number and density across both MS and LS regions. These changes may differ from other domestic pigeon breeds, which could demonstrate changes to neuron number across one subregion and not another depending on behavioural selection type. Repeating this study using staining protocols that differentiate MS and LS regions may further explain differences in septum neuron number, neuron density, and/or volume among pigeon breeds.

Table 3.1. Descriptive statistics for four measurements of anatomy across seven pigeon breeds (n = 39). Measurements include body and brain mass (in g), and telencephalon and septum volume (in mm^3). All values are reported by breed average (mean) and standard deviation (SD).

Breed	n	Body mass (g)		Brain mass (g)		Telencephalon volume (mm^3)		Septum volume (mm^3)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Feral	7	410	56.5	2.20	0.17	991.6	143.3	10.8	1.57
Homer	8	524	37.8	2.28	0.13	989.1	147.9	10.4	1.04
Highflyer	5	407	13.0	2.00	0.11	908.5	68.4	10.5	1.61
Roller	4	415	55.1	2.04	0.19	914.8	90.3	10.3	1.14
Show roller	4	445	19.2	2.07	0.08	924.3	70.3	13.12	1.41
Show homer	5	569	40.4	2.23	0.13	928.1	75.3	12.8	1.34
Cropper	6	508	54.8	2.45	0.16	1118.7	132.3	14.13	1.55

Table 3.2. Descriptive statistics for two measurements of septum anatomy across seven pigeon breeds (n = 39): neuron number and neuron density (in n/mm^3). All values are reported by breed average (mean) and standard deviation (SD).

Breed	n	Septum neuron number		Septum neuron density (/mm^3)	
		Mean	SD	Mean	SD
Feral	7	202047	73001	19232	8133
Homer	8	337330	125379	32549	11870
Highflyer	5	466317	150363	45275	14632
Roller	4	225684	120630	21851	11860
Show roller	4	468748	97394	36474	10842
Show homer	5	402362	70850	31557	5488
Cropper	6	576098	68458	41687	7533

Table 3.3. Results of a Fisher's one-way analysis of variance (ANOVA) comparing absolute measurements across seven pigeon breeds (n = 39). Data were compared by differences in septum volume (in mm^3), septum neuron number, and density (in n/mm^3). ANOVA results are reported using F-ratios (F), degrees of freedom (df), and p-values, wherein (*) denotes a significant difference among breeds. Tukey's honestly significant difference (HSD) post-hoc tests reveal pairwise differences, and significant relationships are denoted using greater than (>) and less than (<) symbols.

Measurements	F	df	p-value	Tukey HSD
Septum volume	5.51	6, 32	**<0.001***	Norwich cropper > feral, homer, highflyer, roller
Septum neuron number	9.70	6, 32	**<0.001***	Feral < highflyer, show roller, show homer, Norwich cropper Norwich cropper > feral, homer, roller
Septum neuron density	4.80	6, 32	**0.001***	Feral < highflyer, Norwich cropper

Table 3.4. Results of analyses of covariance (ANCOVAs) comparing relative log-transformed measurements of neuroanatomy by breed, covariate, and overall model effects across seven pigeon breeds (n = 39). Septum volume (in mm^3) used telencephalon volume (minus septum) as a covariate and septum neuron number used septum volume as a covariate. ANCOVA results are reported using F-ratios (F), degrees of freedom (df), and p-values, wherein (*) denotes a significant difference among breeds. Tukey's HSD post-hoc tests reveal pairwise differences, and significant relationships are denoted using greater than (>) and less than (<) symbols.

Measurements	Breed effects				Covariate effects				Model		
	F	df	p-value	Tukey HSD	Covariate	F	df	p-value	F	df	p-value
Septum volume	5.02	6, 31	**0.001***	Homer < show roller, show homer, Norwich cropper	Tel volume (– septum)	4.47	1, 31	**0.043***	5.85	7, 31	**<0.001***
Septum neuron number	7.01	6, 31	**<0.001***	Feral < highflyer, show roller, show homer, Norwich cropper Roller < highflyer, Norwich cropper	Septum volume	0.218	1, 31	0.644	8.426	7, 31	**<0.001***

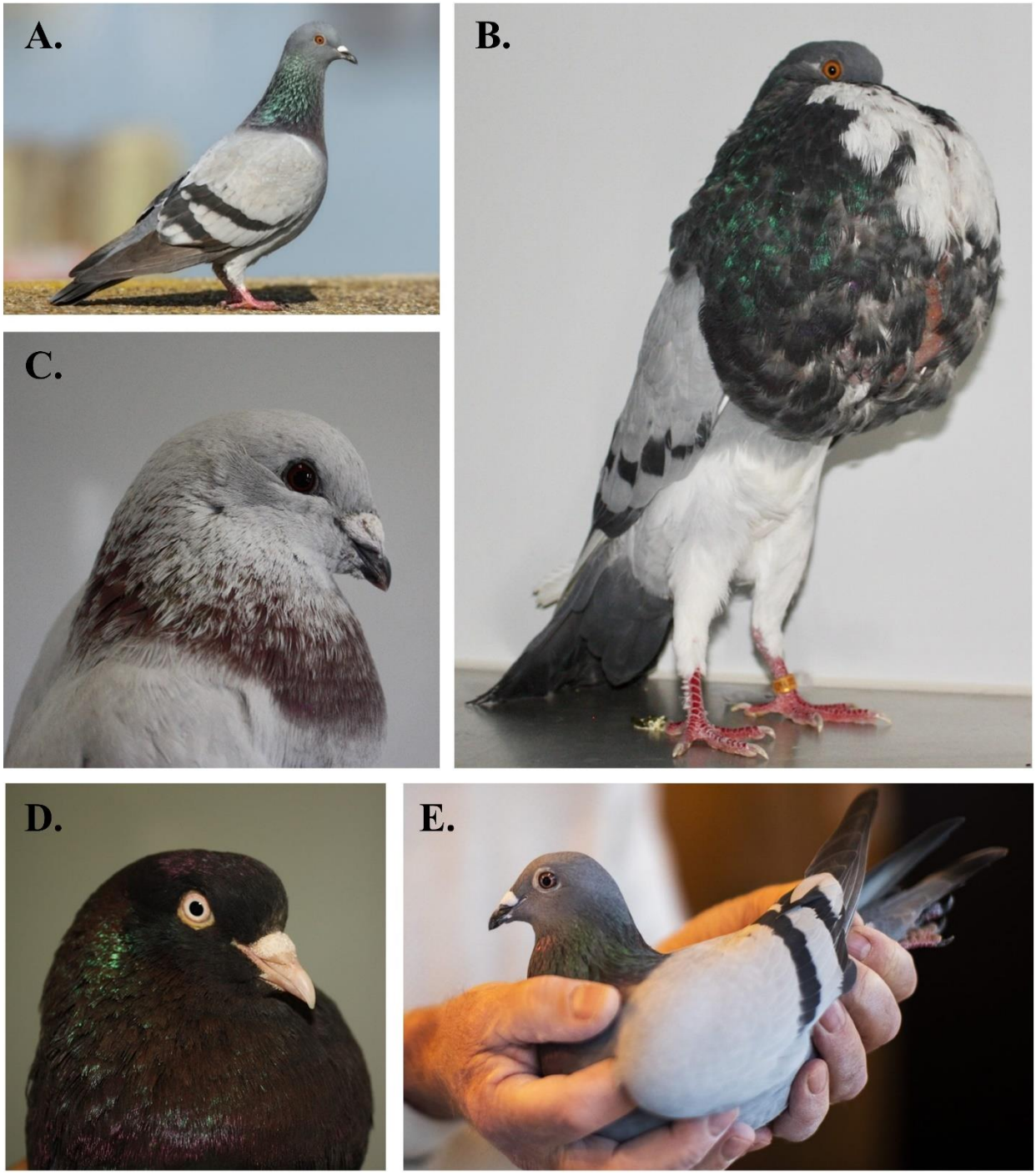

Figure 3.1. Comparison of several breeds of pigeon: A. feral pigeon, descendant of escaped domestic pigeons; B. Norwich cropper, a show breed selected for secondary sexual characters; C. show homer, a show breed selected for morphology, but not homing performance; D. roller, a sporting breed selected for in-flight somersault (rolling) behaviour; and E. racing homer, a sporting breed selected for spatial navigation. Image of homing pigeon courtesy of Francisco Seco (x); image of feral pigeon courtesy of ebird.org (x).

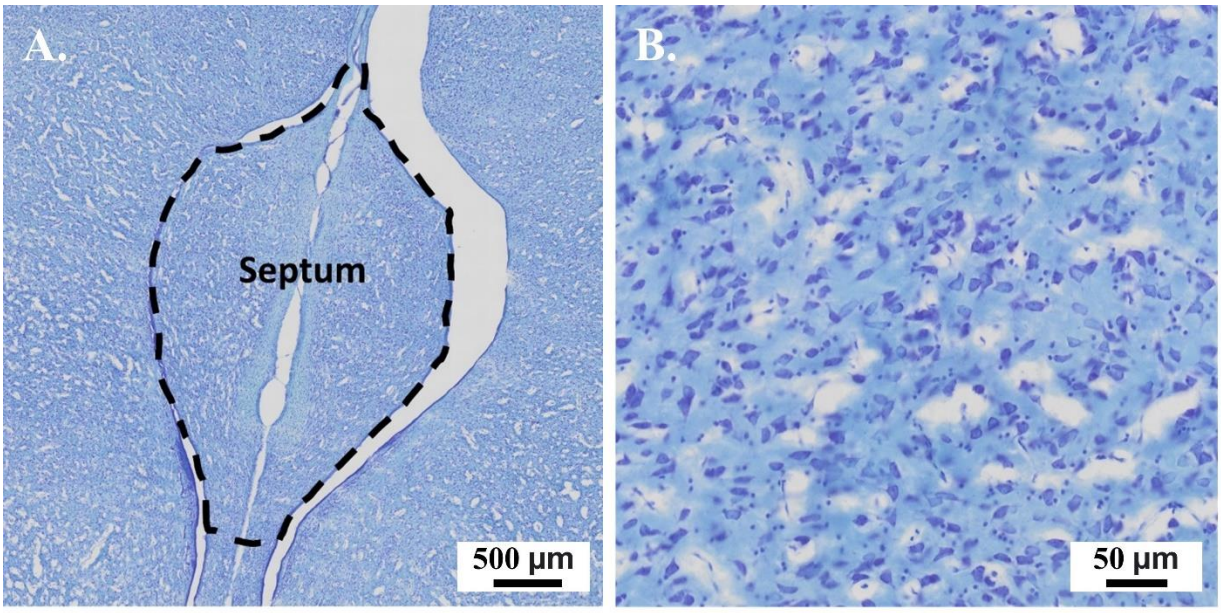

Figure 3.2. Mid-telencephalon sections of homing pigeon septum with cells stained for Nissl bodies using thionin acetate. A. Border outline for septum and B. magnified view of staining within the septum.

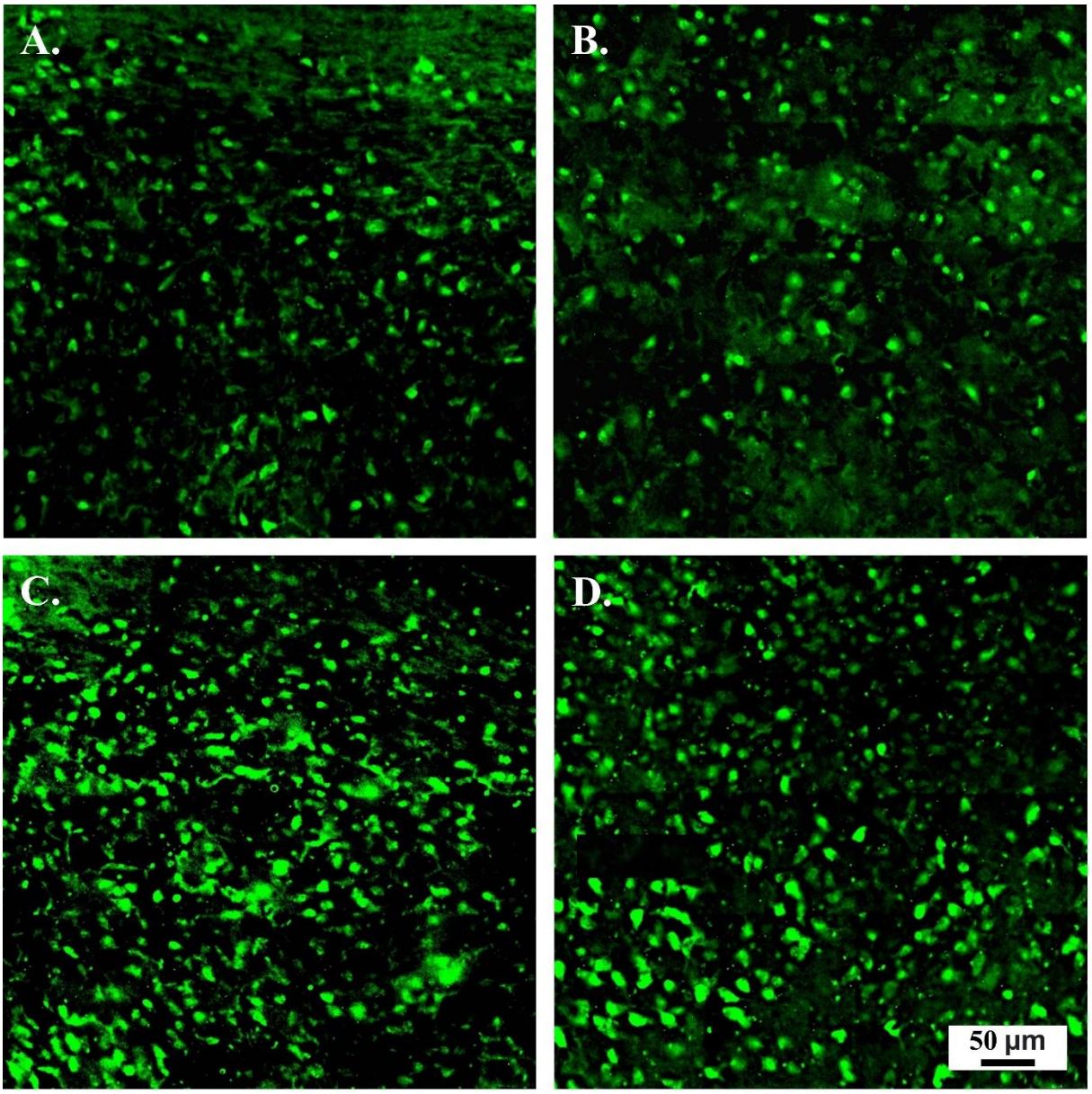

Figure 3.3. Septum neurons immunolabeled for the neuron specific antigen NeuN from the same rostrocaudal location in A. homing; B. feral; C. highflyer; and D. Norwich cropper pigeons.

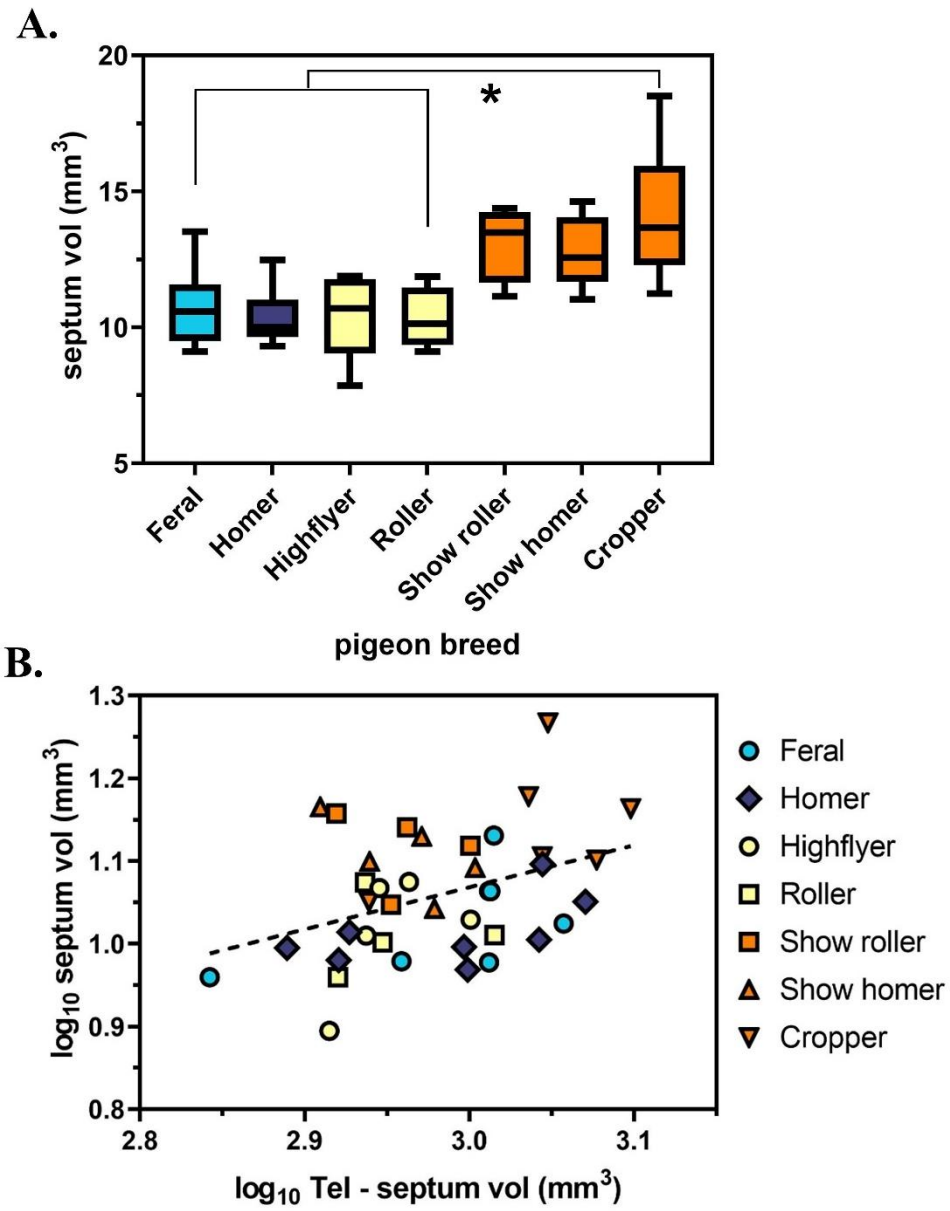

Figure 3.4. Among-breed differences in A. absolute septum volume (mm^3) with min and max range and B. log-transformed septum volume relative to log-transformed telencephalon (minus ROI) volume. As per the legend, ferals = light blue, homers = dark blue, sporting breeds = yellow, show breeds = orange. The dotted line represents the allometric relationship (least-squares linear regression) between x and y across all breeds, and (*) denotes a significant difference from Norwich cropper pigeons ($p < 0.05$).

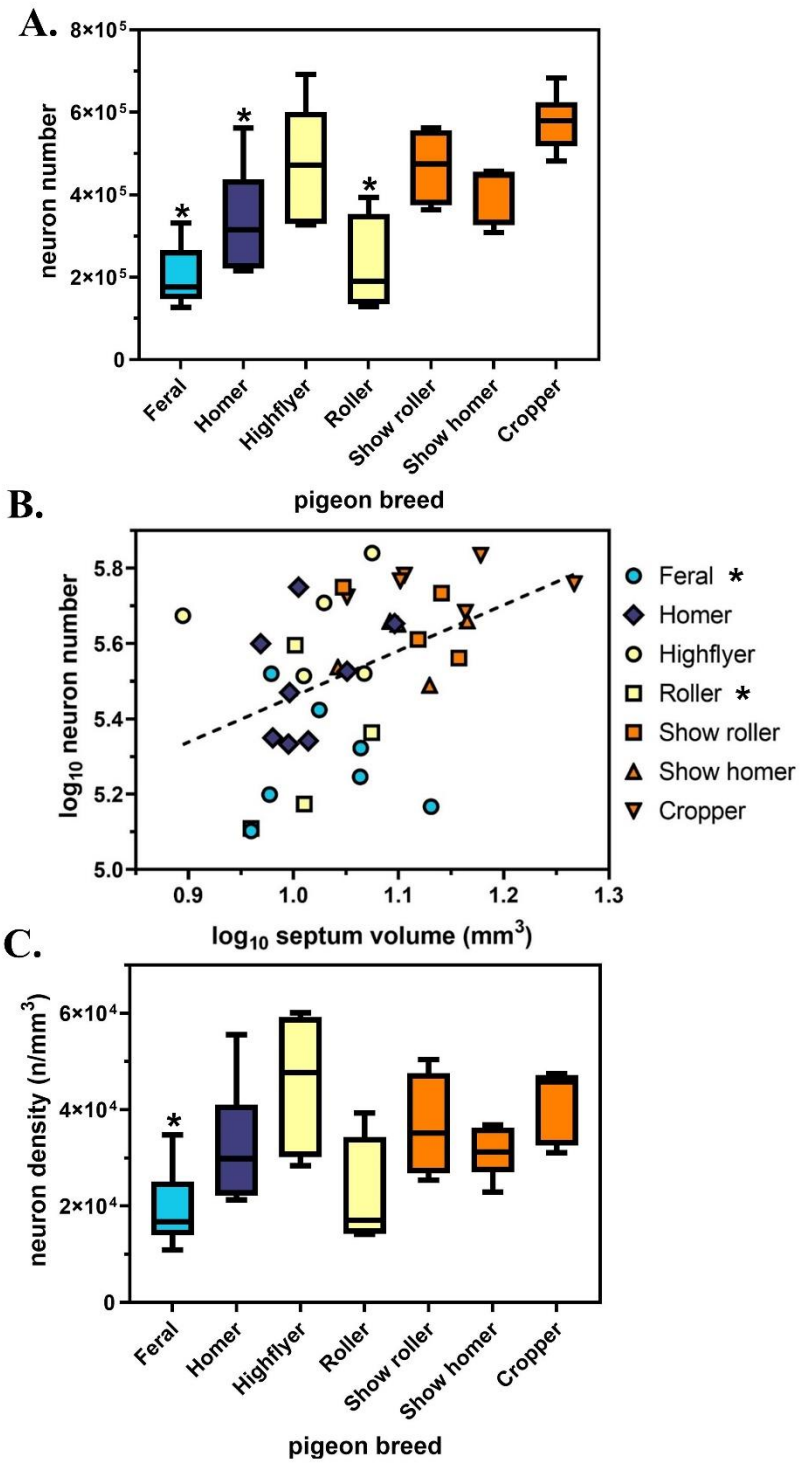

Figure 3.5. Among-breed differences in A. absolute septum neuron number with min and max range; B. log-transformed septum neuron number relative to log-transformed septum volume; and C. absolute neuron density (n/mm^3) with min and max range. As per the legend, ferals = light blue, homers = dark blue, sporting breeds = yellow, show breeds = orange. The dotted line represents the allometric relationship (least-squares linear regression) between x and y across all breeds, and (*) denotes a significant difference from Norwich cropper pigeons ($p < 0.05$).

CHAPTER FOUR: GENERAL DISCUSSION

By using selective breeding techniques, human beings can accelerate selection pressures at an unprecedented rate. For instance, humans have created more variation in domestic dogs (*Canis lupus*) in 30,000 years than natural selection has across all canid species in 45 million years (Francis 2015). The domestic pigeon is no different, and over the last 10,000 years humans have selected Rock Pigeons (*Columba livia*) for diversity in both behaviour and morphology (Price 2002, Shapiro and Domyan 2013). First domesticated as a food source, ceremonial animal, and message bearer (Gilbert and Shapiro 2013), modern pigeon breeds are separated into three categories: utility breeds used for meat production, show breeds that exhibit uniquely exaggerated morphology and/or behaviour, and sporting pigeons that compete in long distance racing, endurance flying, and tumbling competitions. The domestic pigeon displays more variation than any other avian species (Price 2002), and this thesis research aimed to understand how such differential selection for behaviour might cause lasting changes to correlated brain regions.

Behavioural selection

Since as early as 3,000 BC, humans have exploited the homing behaviour of pigeons to carry messages over long distances (Shapiro and Domyan 2013). Birds raised in one city are released in novel locations, and then rely on experience from practice flights as juveniles to navigate to their location of origin. Not all birds would succeed in reaching home, but those that did would survive to reproduce, resulting in selection for improved spatial cognition across generations. Since then, homing pigeons have been used for long-distance racing, disaster relief, and the relay of messages during nearly every war throughout history, often receiving national recognition for their efforts (*Appendix 4*) (Parrott-Holden 1985). As a consequence of selection,

the 80 km homing range of the wild Rock Pigeon (Alleva et al. 1975) has been expanded to as far as 1,000 km in domestic homing pigeons (Degner and Blechman 2011, Gilbert and Shapiro 2013, Shapiro and Domyan 2013). This incredible navigation ability raises the question of *how do they do it?*

Part of the answer to this question can be found in the brain. In general, domestic animals have experienced a regression in brain size in comparison to their wildtype ancestors (Kruska 1988, Kruska 2005), likely caused by decreased reliance on complex behaviours no longer required by captive animals (Agnvall et al. 2017). However, not all behaviours have been simplified in domestic species, and the homing behaviour of domestic homing pigeons has instead been enhanced. In their formative theory of the hippocampus as a cognitive map, O'keefe and Nadel (1978) proposed hippocampal formation (HF), comprising hippocampus (Hp) and its associated structures, as the substrate for spatial behaviour. The theory originated from the finding that single "place" cells fire in Hp of laboratory rats in response to specific locations in their environment (O'Keefe 1979). In pigeons, similar "location" cells have been found in HF (Hough 2022) alongside "path" cells that fire according to familiar visual landmarks, and "pattern" cells that fire in a distance-dependent manner (Hough 2022). The cellular activity of HF proposes a role in navigating through space (Ben-Yishay et al. 2021, Hough 2022). The theory is supported by many other lines of research, including findings that spatial behaviour is impaired when HF is damaged (Bingman et al. 1990, Strasser et al. 1998) and that HF is larger in species with higher dependence on spatial attributes for food storage or locating mates (Sherry et al. 1992). It is therefore logical to hypothesise that HF may play a role in the homing ability of pigeons (Herold et al. 2014), and that is why we predicted this region to be adaptively specialised for improved cognition.

"Adaptive specialisation" theorises that differences in behavioural complexity may be reflected by corresponding changes to the brain regions that control them, e.g., region volume, neuron number, neuron size (Sherry et al. 1992, Roth et al. 2010). The neuroanatomical features that underlie improvements to homing ability have not been previously identified, but the results of our research suggest neuron number is the most accurate marker of differences in spatial cognition. Increasing HF neuron number is a marker of improved performance in spatial tasks (Gould et al. 2013, Pravosudov and Roth 2013, Kverkova et al. 2022), likely by integrating the neural networks (Roth et al. 2010) involved in navigation behaviours. Therefore, a bird bred for improved spatio-cognitive performance might also demonstrate correlated increases to HF neuron number. Homing pigeons demonstrate little difference in HF and subregion (Hp, APH) volume, whereas changes to cognition were more substantially accompanied by large increases in HF neuron number and density. Such an increase is likely the result of positively selecting for genes involved in HF neuron proliferation and survivorship (Shao et al. 2020). We also predicted that avian septum, highly interconnected with HF (Atoji and Wild 2006), might have a role in spatial navigation (Peterson and Bingman 2011) that would result in a similar regional trend in septum. Contrary to our prediction, relative septum volume was lower in homing pigeons compared with most other breeds, and they displayed no differences in septum neuron number or density. Improvements to spatial navigation performance might therefore not be reflected in the neuroanatomical features of septum analysed in this dataset. Septum may instead have a role in spatial working memory processes (Shiflett et al. 2002) that might facilitate some, but not all, aspects of spatial navigation (Peterson and Bingman 2011). For instance, familiar landmark recognition might benefit from improvements to spatial working memory that can be reflected in septum neuroanatomy (Shiflett et al. 2002). However, spatial working memory and spatial

navigation are not equivalent processes, and the former does not inform all navigation methods used during homing (Bingman and Ewry 2020). These results suggest selection for homing has resulted in concomitant increases in HF neuron number, a change that has occurred independently of other brain structures such as septum. However, we also recognise that this trend is not consistent across all domestic breeds used in this study and propose several explanations for this observation.

Homing pigeons have little difference in HF neuroanatomy from show homer and show roller pigeons, despite presumably being better spatial navigators. These recently-derived show breeds are selected for conformation instead of homing or rolling performance (Woodfield and Chambers 1892), but are often crossbred with homing pigeons, among others, for desired traits. Likely, constant breed introgression has impeded potential differences in genetic factors that could result in interbreed differences in HF neuroanatomy. In support of this, most show homers will still home if trained properly (Woodfield and Chambers 1892), suggesting the homing behaviour has not been lost entirely. The influence of breed introgression is further supported by other results from our study. Old Dutch capuchine pigeons are one of the oldest show breeds, first brought to Holland from India in the 15th-century (Parrott-Holden 1985). Known for their unique head crest feathers, capuchines have undergone centuries of divergent selection from homing pigeons and as such have experienced little breed introgression. As a result, there are significant differences in HF neuron number and density between capuchines and homing pigeons not found in breeds that are often crossed.

A breed that does not follow this trend is the Norwich cropper pigeon. Despite being an older breed that is morphologically distinct from homing pigeons, we found little difference in homer and cropper HF neuroanatomy. However, we believe this finding can be explained by the

behavioural selection experienced by cropper pigeons. Historically, croppers were used to steal female pigeons during thieving competitions or directly from competitors' lofts (Hiatt and Esposito 2000). As such, croppers are selected for exaggerated secondary sexual characters that make them desirable to mates, e.g., upright posture, enlarged crop, and constant performance of sexual displays (Hiatt and Esposito 2000). Sexual displays, like most behavioural responses, are regulated by the limbic system (Kolb et al. 2001) which includes HF, hypothalamus, amygdala, and septum (Szekely 1999). HF provides emotional context to sensory inputs and informs social hierarchies (Corrales Parada et al. 2021), whereas septum is likely to modulate agonistic and courtship behaviours (Viglietti-Panzica et al. 1992, Goodson et al. 2005, Taziaux et al. 2006), both of which are elements of sexual displays (Fusani 2008). If sexual displays are controlled by the limbic system, then one might expect concurrent changes reflected in limbic region neuroanatomy (Sherry et al. 1992) in breeds selected for such traits. In accordance with this prediction, Norwich croppers display comparable HF neuron counts and densities to homing pigeons and have larger septa with more neurons than most other domestic pigeon breeds. Similar to selection for spatial cognition in homing pigeons (Shao et al. 2020), selection for sexual characters may have positively selected for genes involved in neuron proliferation and survival across limbic system regions, including HF and septum.

Stress effects

Feral pigeons are a relatively recent introduction to North America (approx. 400 years) (Gilbert and Shapiro 2013), and are descendant of and continue to breed with escaped domestics (Darwin 1859, 1894). While feral pigeons are no longer subject to artificial selection pressures, they experience natural selective pressures including threats of predation, variable weather conditions, and food availability. Feral pigeons are most genetically similar to racing homer

breeds due to their frequent displacement during racing events (Stringham et al. 2012). Despite this, feral pigeons exhibit significantly lower absolute and relative HF and septum neuron numbers and densities than most domestic breeds, including homing pigeons. This finding is most likely explained by the stress of inhabiting urban environments. While captive domestic pigeons have shelter and food provided *ad libitum*, feral birds are subject to food insecurity and constant threats, e.g., predators, vehicles, traps/poisons.

Chronic prolonged stress can reduce the survival of new neurons in avian HF (Robertson et al. 2017, Smulders 2017, Gualtieri et al. 2019) and may also contribute to reductions in neurogenesis observed in wild birds kept in captivity (Sherry and MacDougall-Shackleton 2015). While stress effects have not been extensively documented in septum, septal neurons increase activity in response to both stressful stimuli (Muigg et al. 2007) and social situations (Goodson et al. 2005, Muigg et al. 2007), suggesting stress susceptibility at the neuronal level. Accordingly, we found decreases in neuron number across both HF and septal regions in feral pigeons. While stress negatively influences HF neurogenesis and neuron survivorship (Sherry et al. 1989, Robertson et al. 2017), it remains unclear if a similar trend in septum neurons would be caused by stress directly or by its interconnectivity with HF (Atoji and Wild 2006). A decrease in HF neuron number could negatively influence the formation and maintenance of neural pathways from and to septum (Shors et al. 2012), resulting in correlated decreases in septal neurons. Nonetheless, it is clear that any genetic predisposition for increased neuron number or density (Shao et al. 2020) caused by genetic overlap with homing pigeons (Stringham et al. 2012) is negated by the deleterious effects of chronic stress.

Interestingly, while feral pigeons display significant decreases in HF neuron number, there is little visible difference in neuron number across the rest of the telencephalon (*Appendix*

5), meaning this trend may not be reflected across all brain regions. Since energy is finite and maintaining brain tissue/cells is metabolically costly (Jacobs 1996), differences in resource availability between domestic and feral pigeons could alter the amount of metabolic energy available for the development and maintenance of neural tissue (Lesch et al. 2022). Instead of regional specialisations, feral pigeons might sustain a moderate number of neurons across a variety of brain regions in control of survival-based behaviours. For instance, the nidopallium is a telencephalic region involved in executive functioning, e.g., cognitive flexibility and response regulation (Güntürkün 2005, Atoji and Wild 2009, Nieder 2017), which might be of higher importance to feral pigeons than domestic breeds. Such a finding would be supported by feral pigeon ecology: feral pigeons are habitat generalists with the ability to survive across a range of habitats and environmental conditions (Rose et al. 2006), leading to their coexistence with humans on nearly every continent (Shapiro and Domyan 2013). More research is needed to understand the full influence of stress on neuroanatomy, but differences in neuron number in HF and septum may not be universal across telencephalic structures.

Conclusions

Despite its reputation as a "pest" species, the domestic pigeon is an incredible example of the influence of artificial selection. While early findings suggest domestication can regress brain and brain region size (Kruska 1988, Kruska 2005), we have shown that artificial selection for behaviour can positively influence neuron number in correlated regions of the brain. Notably, homing and Norwich cropper pigeons display regional increases in neuron number and density corresponding to their respectively selected behaviours. Future research could explore the role of experience on neuron proliferation and survivorship in avian telencephalic structures by raising a variety of active and show breeds under identical conditions and/or training regimes. To our

knowledge, this is the first evidence of artificial selection driving specific regional changes in neuron number and density in a domestic species.

REFERENCES

Agnvall, B., J. Belteky, and P. Jensen. 2017. Brain size is reduced by selection for tameness in Red Junglefowl- correlated effects in vital organs. Sci Rep **7**:3306.

Alleva, E., N. E. Baldaccini, A. Foà, and E. Visalberghi. 1975. Homing behaviour of the Rock pigeon. Monit Zool Ital **9**:213-224.

Altunkaynak, B. Z., M. E. Onger, M. E. Altunkaynak, E. Ayrancı, and S. Canan. 2011. A brief introduction to stereology and sampling strategies: basic concepts of stereology. NeuroQuantology **10**:31-43.

Atoji, Y., and J. M. Wild. 2006. Anatomy of the avian hippocampal formation. Rev Neurosci **17**:3-15.

Atoji, Y., and J. M. Wild. 2009. Afferent and efferent projections of the central caudal nidopallium in the pigeon (*Columba livia*). J Comp Neurol **517**:350-370.

Atwi, S., D. McMahon, H. Scharfman, and N. J. MacLusky. 2016. Androgen modulation of hippocampal structure and function. Neuroscientist **22**:46-60.

Baptista, L. F., J. E. Martínez gómez, and H. M. Horblit. 2009. Darwin's pigeons and the evolution of the columbiformes: recapitulation of ancient genes. Acta Zool Mex **25**:719-741.

Ben-Yishay, E., K. Krivoruchko, S. Ron, N. Ulanovsky, D. Derdikman, and Y. Gutfreund. 2021. Directional tuning in the hippocampal formation of birds. Curr Biol **31**:2592-2602 e2594.

Biała, A., A. Dybus, E. Pawlina, and W. Proskura. 2015. Genetic diversity in eight pure breeds and urban form of domestic pigeon (*Columba livia* var. domestica) based on seven microsatellite loci. J Anim Plant Sci **25**:1741-1745.

Bingman, V. P. 2018. Requiem for a heavyweight - can anything more be learned from homing pigeons about the sensory and spatial-representational basis of avian navigation? J Exp Biol **221**:jeb163089.

Bingman, V. P., and E. M. Ewry. 2020. On a search for a neurogenomics of cognitive processes supporting avian migration and navigation. Integr Comp Biol **60**:967-975.

Bingman, V. P., A. Gagliardo, G. E. Hough, 2nd, P. Ioale, M. C. Kahn, and J. J. Siegel. 2005. The avian hippocampus, homing in pigeons and the memory representation of large-scale space. Integr Comp Biol **45**:555-564.

Bingman, V. P., G. E. Hough, 2nd, M. C. Kahn, and J. J. Siegel. 2003. The homing pigeon hippocampus and space: in search of adaptive specialization. Brain Behav Evol **62**:117-127.

Bingman, V. P., P. Ioale, G. Casini, and P. Bagnoli. 1990. The avian hippocampus: evidence for a role in the development of the homing pigeon navigational map. Behav Neurosci **104**:906-911.

Bolhuis, J. J., and E. M. Macphail. 2001. A critique of the neuroecology of learning and memory. Trends Cogn Sci **5**:426-433.

Brady, J. V., and W. J. Nauta. 1953. Subcortical mechanisms in emotional behavior: affective changes following septal forebrain lesions in the albino rat. J Comp Physiol Psychol **46**:339-346.

Burley, N., and N. Moran. 1979. The significance of age and reproductive experience in the mate preferences of feral pigeons, *Columba livia*. Anim Behav **27**:686-698.

Cao, J., K. Wenberg, and M.-F. Cheng. 2002. Lesion induced new neuron incorporation in the adult hypothalamus of the avian brain. Brain Res J **943**:80-92.

Chancellor, L. V., T. C. Roth, L. D. LaDage, and V. V. Pravosudov. 2011. The effect of environmental harshness on neurogenesis: a large-scale comparison. Dev Neurobiol **71**:246-252.

Cnotka, J., M. Mohle, and G. Rehkamper. 2008. Navigational experience affects hippocampus size in homing pigeons. Brain Behav Evol **72**:233-238.

Coppola, V. J., N. Kanyok, A. J. Schreiber, M. E. Flaim, and V. P. Bingman. 2016. Changes in hippocampal volume and neuron number co-occur with memory decline in old homing pigeons (*Columba livia*). Neurobiol Learn Mem **131**:117-120.

Coppola, V. J., D. Nardi, and V. P. Bingman. 2021. Age-associated decline in septum neuronal activation during spatial learning in homing pigeons (*Columba livia*). Behav Brain Res **397**:112948.

Corrales Parada, C. D., A. Morandi-Raikova, O. Rosa-Salva, and U. Mayer. 2021. Neural basis of unfamiliar conspecific recognition in domestic chicks (*Gallus gallus domesticus*). Behav Brain Res **397**:112927.

Daniels, T. J., and M. Bekoff. 1989. Feralization: The making of wild domestic animals. Behav Processes **19**:79-94.

Darwin, C. 1859. On the origin of species by means of natural selection, or preservation of favoured races in the struggle for life. London : John Murray, 1859.

Darwin, C. 1894. The variation of animals and plants under domestication. 2nd edition. D. Appleton and Co., New York, New York, USA.

Day, L. B., M. Guerra, B. A. Schlinger, and S. I. Rothstein. 2008. Sex differences in the effects of captivity on hippocampus size in brown-headed cowbirds (*Molothrus ater obscurus*). Behav Neurosci **122**:527-534.

Degner, D., and A. D. Blechman. 2011. Cairo's Fancy Fliers. Saudi Aramco World.

den Boer-Visser, A., M. Brittijn, and J. L. Dubbeldam. 2004. A stereotaxic atlas of the brain of the collared dove, *Streptopelia decaocto*. Shaker Publishing, Maastricht, Limburg, Netherlands.

Ebinger, P. 1974. A cytoarchitectonic volumetric comparison of brains in wild and domestic sheep. Z Anat Entwicklungsgesch **144**:267-302.

Ebinger, P. 1995. Domestication and plasticity of brain organization in mallards (Anas platyrhynchos). Brain Behav Evol **45**:286-300.

Ebinger, P., and R. Löhmer. 1984. Comparative quantitative investigations on brains of rock doves, domestic and urban pigeons (*Columba livia*). J Zool Syst Evol Res **22**:136-145.

Edrich, W., and W. T. Keeton. 1977. A comparison of homing behavior in feral and homing pigeons. Z Tierpsychol **44**:389-401.

Epstein, R. A., E. Z. Patai, J. B. Julian, and H. J. Spiers. 2017. The cognitive map in humans: spatial navigation and beyond. Nat Neurosci **20**:1504-1513.

Erickson, K. I., M. W. Voss, R. S. Prakash, C. Basak, A. Szabo, L. Chaddock, J. S. Kim, S. Heo, H. Alves, S. M. White, T. R. Wojcicki, E. Mailey, V. J. Vieira, S. A. Martin, B. D. Pence, J. A. Woods, E. McAuley, and A. F. Kramer. 2011. Exercise training increases size of hippocampus and improves memory. Proc Natl Acad Sci U S A **108**:3017-3022.

Frahm, H. D., and G. Rehkamper. 1998. Allometric comparison of the brain and brain structures in the white crested polish chicken with uncrested domestic chicken breeds. Brain Behav Evol **52**:292-307.

Francis, R. C. 2015. Domesticated: evolution in a man-made world. W. W. Norton & Company, New York, New York, USA.

Freas, C. A., T. C. Roth, L. D. LaDage, V. V. Pravosudov, and J. Grindstaff. 2013. Hippocampal neuron soma size is associated with population differences in winter climate severity in food-caching chickadees. Funct Ecol **27**:1341-1349.
Fusani, L. 2008. Testosterone control of male courtship in birds. Horm Behav **54**:227-233.
Gagliardo, A., P. Ioale, M. Savini, H. P. Lipp, and G. Dell'Omo. 2007. Finding home: the final step of the pigeons' homing process studied with a GPS data logger. J Exp Biol **210**:1132-1138.
Gallistel, C. R. 1990. The organization of learning. The MIT Press, Cambridge, Massachusetts, USA.
Gilbert, M. T. P., and M. D. Shapiro. 2013. Pigeons, domestication of. Pages 1-5 *in* Encyclopedia of Global Archaeology. Springer Science & Business Media, New York, New York, USA.
Goodson, J. L., R. Eibach, J. Sakata, and E. Adkins-Regan. 1998. The morphEffect of septal lesions on male song and aggression in the colonial zebra finch (*Taeniopygia guttata*) and the territorial field sparrow (Spizella pusilla). Behav Brain Res **98**:167-180.
Goodson, J. L., A. K. Evans, and L. Lindberg. 2004. Chemoarchitectonic subdivisions of the songbird septum and a comparative overview of septum chemical anatomy in jawed vertebrates. J Comp Neurol **473**:293-314.
Goodson, J. L., A. K. Evans, L. Lindberg, and C. D. Allen. 2005. Neuro-evolutionary patterning of sociality. Proc Biol Sci **272**:227-235.
Gould, K. L., K. E. Gilbertson, A. J. Hrvol, J. C. Nelson, A. L. Seyfer, R. M. Brantner, and A. C. Kamil. 2013. Differences in relative hippocampus volume and number of hippocampus neurons among five corvid species. Brain Behav Evol **81**:56-70.
Gualtieri, F., E. A. Armstrong, G. K. Longmoor, R. B. D'Eath, V. Sandilands, T. Boswell, and T. V. Smulders. 2019. Unpredictable chronic mild stress suppresses the incorporation of new neurons at the caudal pole of the chicken hippocampal formation. Sci Rep **9**:7129.
Güntürkün, O. 2005. The avian 'prefrontal cortex' and cognition. Curr Opin Neurobiol **15**:686-693.
Healy, S. D., E. Gwinner, and J. R. Krebs. 1996. Hippocampal volume in migratory and non-migratory warblers: effects of age and experience. Behav Brain Res **81**:61-68.
Herculano-Houzel, S. 2018. Embodied (embrained?) cognitive evolution, at last! Comp Cogn Behav **13**:91-94.
Herold, C., V. P. Bingman, F. Strockens, S. Letzner, M. Sauvage, N. Palomero-Gallagher, K. Zilles, and O. Gunturkun. 2014. Distribution of neurotransmitter receptors and zinc in the pigeon (*Columba livia*) hippocampal formation: A basis for further comparison with the mammalian hippocampus. J Comp Neurol **522**:2553-2575.
Herold, C., V. J. Coppola, and V. P. Bingman. 2015. The maturation of research into the avian hippocampal formation: Recent discoveries from one of the nature's foremost navigators. Hippocampus **25**:1193-1211.
Hiatt, S., and D. Esposito. 2000. The pigeon guide. Silvio Mattacchione and Company, Port Perry, Ontario, Canada.
Hough, G. E. 2022. Neural substrates of homing pigeon spatial navigation: results from electrophysiology studies. Front Psychol **13**:867939.
Howard, V., and M. Reed. 2004. Unbiased Stereology. Garland Science, New York, New York, USA.
Jacobs, L. F. 1996. Sexual selection and the brain. Trends in Ecology & Evolution **11**:82-86.

Jacobs, L. F. 2003. The evolution of the cognitive map. Brain Behav Evol **62**:128-139.
Jerison, H. J. 1973. Evolution of the Brain and Intelligence. Academic Press, New York, New York, USA.
Jerolmack, C. 2007. Animal archeology: Domestic pigeons and the nature-culture dialectic. Qual Sociol Rev **3**:74-95.
Karten, H. J., and W. Hodos. 1967. A stereotaxic atlas of the brain of the pigeon (*Columba livia*). The Johns Hopkins Press, Baltimore.
Keefe, A. 2017. Inside the Quirky World of Competitive Pigeon Seduction. National Geographic. https://nationalgeographic.com/photography/article/doo-pigeons-flying-scotland.
Kolb, B., I. Q. Whishaw, and G. C. Teskey. 2001. An introduction to brain and behavior. Worth Publishers, New York, New York, USA.
Krebs, J. R., D. F. Sherry, S. D. Healy, V. H. Perry, and A. L. Vaccarino. 1989. Hippocampal specialization of food-storing birds. Proc Natl Acad Sci U S A **86**:1388-1392.
Kruska, D. 1988. Effects of domestication on brain structure and behavior in mammals. Hum Evol **3**:473-485.
Kruska, D. C. 2005. On the evolutionary significance of encephalization in some eutherian mammals: effects of adaptive radiation, domestication, and feralization. Brain Behav Evol **65**:73-108.
Kverkova, K., L. Marhounova, A. Polonyiova, M. Kocourek, Y. Zhang, S. Olkowicz, B. Strakova, Z. Pavelkova, R. Vodicka, D. Frynta, and P. Nemec. 2022. The evolution of brain neuron numbers in amniotes. Proc Natl Acad Sci U S A **119**:e2121624119.
Lesch, R., K. Kotrschal, A. C. Kitchener, W. T. Fitch, and A. Kotrschal. 2022. The expensive-tissue hypothesis may help explain brain-size reduction during domestication. Commun Integr Biol **15**:190-192.
Lubar, J. F., and R. Numan. 1973. Behavioral and physiological studies of septal function and related medial cortical structures. Behav Biol **8**:1-25.
Mehlhorn, J., and G. Rehkamper. 2009. Neurobiology of the homing pigeon--a review. Naturwissenschaften **96**:1011-1025.
Metzdorf, R., M. Gahr, and L. Fusani. 1999. Distribution of aromatase, estrogen receptor, and androgen receptor mRNA in the forebrain of songbirds and nonsongbirds. J Comp Neurol **407**:115-129.
Montagnese, C. M., A. D. Szekely, A. Adam, and A. Csillag. 2004. Efferent connections of septal nuclei of the domestic chick (*Gallus domesticus*): an anterograde pathway tracing study with a bearing on functional circuits. J Comp Neurol **469**:437-456.
Mowrer, O. H. 1940. The tumbler pigeon. J Comp Psychol **30**:515-533.
Muigg, P., U. Hoelzl, K. Palfrader, I. Neumann, A. Wigger, R. Landgraf, and N. Singewald. 2007. Altered brain activation pattern associated with drug-induced attenuation of enhanced depression-like behavior in rats bred for high anxiety. Biol Psychiatry **61**:782-796.
Nieder, A. 2017. Inside the corvid brain—probing the physiology of cognition in crows. Curr Opin Behav Sci **16**:8-14.
O'Keefe, J. 1979. A review of the hippocampal place cells. Prog Neurobiol **13**:419-439.
O'keefe, J., and L. Nadel. 1978. The hippocampus as a cognitive map. Oxford Clarendon Press, Oxford, England.

O'Rourke, T., P. T. Martins, R. Asano, R. O. Tachibana, K. Okanoya, and C. Boeckx. 2021. Capturing the Effects of Domestication on Vocal Learning Complexity. Trends Cogn Sci **25**:462-474.

Okanoya, K. 2004. The Bengalese finch: a window on the behavioral neurobiology of birdsong syntax. Ann N Y Acad Sci **1016**:724-735.

Parrott-Holden, J. 1985. In Praise of Pigeons. AFA Watchbird **12**:30-33.

Peterson, R. M., and V. P. Bingman. 2011. Septal area lesions impair spatial working memory in homing pigeons (*Columba livia*). Neurobiol Learn Mem **96**:353-360.

Pravosudov, V. V., and N. S. Clayton. 2002. A test of the adaptive specialization hypothesis: Population differences in caching, memory, and the hippocampus in black-capped chickadees (*Poecile atricapilla*). Behav Neurosci **116**:515-522.

Pravosudov, V. V., and T. C. Roth. 2013. Cognitive ecology of food hoarding: the evolution of spatial memory and the hippocampus. Annu Rev Ecol Evol Syst **44**:173-193.

Price, E. O. 1984. Behavioral aspects of animal domestication. Q Rev Biol **59**:1-32.

Price, T. D. 2002. Domesticated birds as a model for the genetics of speciation by sexual selection. Genetica **116**:311-327.

Puelles, L., M. Martinez-de-la-Torre, S. Martinez, C. Watson, and G. Paxinos. 2018. The chick brain in stereotaxic coordinates and alternate stains: Featuring neuromeric divisions and mammalian homologies. Academic Press, New York, New York, USA.

Ramirez, J. M., C. Salas, and M. Portavella. 1988. Offense and defense after lateral septal lesions in *Columba livia*. Int J Neurosci **41**:241-250.

Rehkamper, G., H. D. Frahm, and J. Cnotka. 2008. Mosaic evolution and adaptive brain component alteration under domestication seen on the background of evolutionary theory. Brain Behav Evol **71**:115-126.

Rehkamper, G., E. Haase, and H. D. Frahm. 1988. Allometric comparison of brain weight and brain structure volumes in different breeds of the domestic pigeon, *Columba livia f.d.* (fantails, homing pigeons, strassers). Brain Behav Evol **31**:141-149.

Reiner, A., D. J. Perkel, L. L. Bruce, A. B. Butler, A. Csillag, W. Kuenzel, L. Medina, G. Paxinos, T. Shimizu, G. Striedter, M. Wild, G. F. Ball, S. Durand, O. Gunturkun, D. W. Lee, C. V. Mello, A. Powers, S. A. White, G. Hough, L. Kubikova, T. V. Smulders, K. Wada, J. Dugas-Ford, S. Husband, K. Yamamoto, J. Yu, C. Siang, E. D. Jarvis, and F. Avian Brain Nomenclature. 2004. Revised nomenclature for avian telencephalon and some related brainstem nuclei. J Comp Neurol **473**:377-414.

Robertson, B. A., L. Rathbone, G. Cirillo, R. B. D'Eath, M. Bateson, T. Boswell, P. W. Wilson, I. C. Dunn, and T. V. Smulders. 2017. Food restriction reduces neurogenesis in the avian hippocampal formation. PLoS One **12**:e0189158.

Rose, E., P. Nagel, and D. Haag-Wackernagel. 2006. Spatio-temporal use of the urban habitat by feral pigeons (*Columba livia*). Behav Ecol Sociobiol **60**:242-254.

Roth, T. C., 2nd, A. Brodin, T. V. Smulders, L. D. LaDage, and V. V. Pravosudov. 2010. Is bigger always better? A critical appraisal of the use of volumetric analysis in the study of the hippocampus. Philos Trans R Soc Lond B Biol Sci **365**:915-931.

Roth, T. C., and V. V. Pravosudov. 2009. Hippocampal volumes and neuron numbers increase along a gradient of environmental harshness: a large-scale comparison. Proc Biol Sci **276**:401-405.

Schlinger, B. A., and C. J. Saldanha. 2005. Songbirds: A novel perspective on estrogens and the aging brain. Age **27**:287-296.

Shao, Y., H. Y. Tian, J. J. Zhang, H. Kharrati-Koopaee, X. Guo, X. L. Zhuang, M. L. Li, H. A. Nanaie, E. Dehghani Tafti, B. Shojaei, M. Reza Namavar, N. Sotoudeh, A. Oluwakemi Ayoola, J. L. Li, B. Liang, A. Esmailizadeh, S. Wang, and D. D. Wu. 2020. Genomic and phenotypic analyses reveal mechanisms underlying homing ability in pigeon. Mol Biol Evol **37**:134-148.

Shapiro, M. D., and E. T. Domyan. 2013. Domestic pigeons. Curr Biol **23**:R302-303.

Sherry, D. F. 2006. Neuroecology. Annu Rev Psychol **57**:167-197.

Sherry, D. F., L. F. Jacobs, and S. J. Gaulin. 1992. Spatial memory and adaptive specialization of the hippocampus. Trends Neurosci **15**:298-303.

Sherry, D. F., and S. A. MacDougall-Shackleton. 2015. Seasonal change in the avian hippocampus. Front Neuroendocrinol **37**:158-167.

Sherry, D. F., A. L. Vaccarino, K. Buckenham, and R. S. Herz. 1989. The hippocampal complex of food-storing birds. Brain Behav Evol **34**:308-317.

Shiflett, M. W., K. L. Gould, T. V. Smulders, and T. J. DeVoogd. 2002. Septum volume and food-storing behavior are related in parids. J Neurobiol **51**:215-222.

Shimizu, T., A. N. Bowers, C. A. Budzynski, M. C. Kahn, and V. P. Bingman. 2004. What does a pigeon (*Columba livia*) brain look like during homing? Selective examination of ZENK expression. Behav Neurosci **118**:845-851.

Shors, T. J., M. L. Anderson, D. M. Curlik, 2nd, and M. S. Nokia. 2012. Use it or lose it: how neurogenesis keeps the brain fit for learning. Behav Brain Res **227**:450-458.

Smulders, T. V. 2017. The Avian Hippocampal Formation and the Stress Response. Brain Behav Evol **90**:81-91.

Smulders, T. V., A. D. Sasson, and T. J. DeVoogd. 1995. Seasonal variation in hippocampal volume in a food-storing bird, the black-capped chickadee. J Neurobiol **27**:15-25.

Strasser, R., V. P. Bingman, P. Ioale, G. Casini, and P. Bagnoli. 1998. The homing pigeon hippocampus and the development of landmark navigation. Dev Psychobiol **33**:305-315.

Striedter, G. F. 2015. Evolution of the hippocampus in reptiles and birds. J Comp Neurol **524**:496-517.

Stringham, S. A., E. E. Mulroy, J. Xing, D. Record, M. W. Guernsey, J. T. Aldenhoven, E. J. Osborne, and M. D. Shapiro. 2012. Divergence, convergence, and the ancestry of feral populations in the domestic rock pigeon. Curr Biol **22**:302-308.

Szekely, A. D. 1999. The avian hippocampal formation: subdivisions and connectivity. Behav Brain Res **98**:219-225.

Taziaux, M., C. A. Cornil, C. Dejace, L. Arckens, G. F. Ball, and J. Balthazart. 2006. Neuroanatomical specificity in the expression of the immediate early gene c-fos following expression of appetitive and consummatory male sexual behaviour in Japanese quail. Eur J Neurosci **23**:1869-1887.

The jamovi project. 2022. jamovi (Version 1.6) [Computer Software]. https://jamovi.org/.

Uysal, N., K. Tugyan, B. M. Kayatekin, O. Acikgoz, H. A. Bagriyanik, S. Gonenc, D. Ozdemir, I. Aksu, A. Topcu, and I. Semin. 2005. The effects of regular aerobic exercise in adolescent period on hippocampal neuron density, apoptosis and spatial memory. Neurosci Lett **383**:241-245.

Viglietti-Panzica, C., G. C. Anselmetti, J. Balthazart, N. Aste, and G. C. Panzica. 1992. Vasotocinergic innervation of the septal region in the Japanese quail: sexual differences and the influence of testosterone. Cell Tissue Res **267**:261-265.

Ward, B. J., L. B. Day, S. R. Wilkening, D. R. Wylie, D. M. Saucier, and A. N. Iwaniuk. 2012. Hummingbirds have a greatly enlarged hippocampal formation. Biol Lett **8**:657-659.
Whishaw, I. Q., D. J. Hines, and D. G. Wallace. 2001. Dead reckoning (path integration) requires the hippocampal formation: evidence from spontaneous exploration and spatial learning tasks in light (allothetic) and dark (idiothetic) tests. Behav Brain Res **127**:49-69.
Woodfield, V., and J. Chambers. 1892. The Show Homer Pigeon.

Appendix 1: AGE-RELATED EFFECTS ON HF NEUROANATOMY

Results for a Pearson's correlation matrix of age-related effects on measurements of neuroanatomy across six breeds of domestic pigeon used in Chapter 2 (n = 28; mean = 2.6 ± 0.995 years, 95% CI). Pearson's correlation results are reported using correlation coefficients (Pearson's r) and p-values, wherein (*) denotes a significant correlation between measurement and age.

Measurements	Pearson's r	p-value
Body mass	0.108	0.585
Brain mass	0.259	0.183
Brain (CB removed) volume	0.357	0.062
Telencephalon volume	0.231	0.238
Hippocampus volume	0.608	**<0.001***
Area parahippocampalis volume	0.497	**0.007***
Hippocampal formation volume	0.674	**<0.001***
HF neuron number	0.597	**<0.001***
HF neuron density	0.348	0.070

Appendix 2: SEX-RELATED EFFECTS ON SEPTUM NEUROANATOMY

Results for an independent samples t-test comparing male (n = 14) and female (n = 16) septum neuroanatomy across seven breeds of domestic pigeon used in Chapter 3 (n = 30). T-test results are reported using T-statistics (T), degrees of freedom (df), and p-values, wherein (*) denotes a significant difference between sexes.

Measurements	T-statistic	df	p-value
Septum volume	0.159	28	0.875
Septum neuron number	0.727	28	0.473
Septum neuron density	0.978	28	0.336

Appendix 3: AGE-RELATED EFFECTS ON SEPTUM NEUROANATOMY

Results for a Pearson's correlation matrix of age-related effects on measurements of septum neuroanatomy across five breeds of domestic pigeon used in Chapter 3 (n = 23; mean = 2.8 ± 1.136 years, 95% CI). Pearson's correlation results are reported using correlation coefficients (Pearson's r) and p-values, wherein (*) denotes a significant correlation between measurement and age.

Measurements	Pearson's r	p-value
Septum volume	-0.384	0.070
Septum neuron number	-0.263	0.225
Septum neuron density	-0.048	0.827

Appendix 4: HISTORICAL USE OF HOMING PIGEONS

Cher Ami, recipient of the Croix de Guerre for his efforts during the First World War. A trained homing pigeon who carried a message from the 77th Infantry Division of the United States Army, disclosing their location and entrapment by German forces. En route, Cher Ami was shot through the breast, eye, and leg, but still succeeded in delivering a message responsible for saving 194 soldiers. Image courtesy of Defense Visual Information Distribution Service (x).

Appendix 5: HIPPOCAMPUS OF HOMING AND FERAL PIGEONS

Hippocampal formation immunolabeled for the neuron specific antigen NeuN from the same rostrocaudal location in A. homing and B. feral pigeons.