Investigating the genetic basis of boldness and reproductive performance traits in the grey seal (*Halichoerus grypus*)

by

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Abstract

Individual variation provides the material for natural selection to act upon, influencing ecological and evolutionary processes. Much phenotypic variation in traits, such as behaviour and life-history, have a moderate to high heritable component, suggesting that these traits may have adaptive potential and are genetically influenced. Yet, investigating the genetic basis of complex traits remains limited to few natural systems – partly due to the difficulty of sampling phenotypes in the wild, a lack of existing genomic resources, and detailed pedigree information – and remains a great challenge in biology today. Advances in DNA sequencing technologies now permit investigations into the evolutionary potential of traits as well as the genetic architecture underlying phenotypic variation in the wild. In this dissertation, I examine the genetic basis of maternal performance traits in free-ranging grey seals (*Halichoerus grypus*), a long-lived, iteroparous species that has rebounded following a long history of overexploitation. First, I performed a literature review and meta-analysis to examine trends in analytical molecular approaches used to elucidate the genetic basis of animal behaviour. Analyzing nearly 150 studies focused on candidate gene, quantitative trait locus mapping, and genome-wide association analyses, I discovered evidence of limited taxonomic breadth in the literature. I highlighted commonly studied candidate genes and behaviours, and further reported global genetic effect sizes for each approach undertaken. Second, I determined the existence of an animal personality signal along the shy-bold continuum in the Sable Island National Park Preserve (Nova Scotia, Canada) population of female grey seals. Using behavioural data collected over a nine-year period (2008-2016), I showed that boldness is highly repeatable both between and within years. Boldness was influenced by maternal age, such that younger females were generally less bold than older, more experienced females providing some support for the life-history trade-off

hypothesis. Seal pups produced by bolder females were on average ~2 kg heavier than pups of shy females. Third, I used a candidate gene approach to investigate relationships between genetic variants and repeated measures of boldness, offspring weaning mass, and lactation duration in grey seals. I isolated and re-sequenced five candidate genes commonly screened in primates, rodents, and passerines. Here, I found genetic effects of the serotonin transporter (*SERT*) and dopamine receptor D4 (*DRD4*) genes on boldness and offspring weaning mass. Genotypes explained 6.52-13.66% of total trait variation. Lastly, using a reduced representation DNA sequencing method, I obtained genome-wide genotypic data for over 450 female grey seals and determined that eight maternal traits, representing morphological, life-history, and behavioural traits, had low to moderate heritability (h^2 = 0.08-0.38). Genome-wide association analyses did not reveal any loci that were significantly associated with the traits examined, suggesting these traits are polygenic. Altogether, this thesis integrated molecular genetic methodologies and statistical approaches with a longitudinal field program, and presented support for the adaptive potential of fitness-related traits in Sable Island grey seals. Results further provided insights into factors and mechanisms underlying maternal performance variation in this ecologically important marine predator. Though challenges exist in investigating the genetic basis of quantitative traits, such analyses give insight into the evolutionary dynamics and capacity for adaptation in natural populations, especially relevant as biodiversity is exposed to novel selection pressures and changing environmental conditions.

Preface

This thesis is the product of original work as performed by Christine M. Bubac. However, this work would not have been possible without collaborative efforts and access to Canada's Department of Fisheries and Oceans' (DFO's) extensive longitudinal dataset and archived tissue samples. To reflect this collaboration, the use of "we" is used throughout much of this dissertation. All procedures on grey seals used in this study complied with the guidelines for animal use as provided by the Canadian Council on Animal Care, and were approved by the Animal Care Committees of the University of Alberta and the DFO, Canada.

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A version of Chapter 2 of this thesis has been published as: Bubac CM, Miller JM, Coltman DW. 2020. The genetic basis of animal behavioural diversity in natural populations. Molecular Ecology 29: 1957-1971. I conceived of the study, collected data, analyzed data, and wrote the manuscript. JMM contributed to data analysis and manuscript writing. DWC provided supervisory guidance and editorial comments on the manuscript.

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Table of Contents

Chapter 1: General introduction 1
1.1 Introduction 1
1.1.1 Individual variation 1
1.1.2 Behavioural variation and animal personality 1
1.1.3 Genetic basis of phenotypic variation 2
1.1.4 DNA sequencing technologies 3
1.1.5 Marine mammals 4
1.1.6 Grey seals 5
1.1.7 Longitudinal studies: Sable Island 6
1.2 Thesis objectives and data chapters 6
1.3 References 9

Chapter 2: The genetic basis of animal behavioural diversity in natural populations 14
2.1 Abstract 14
2.2 Introduction 14
2.3 Methods 18
2.3.1 Literature search and inclusion criteria 18
2.3.2 Data extraction 20
2.3.3 Data analysis 21
2.4 Results 22
2.4.1 Taxonomic spread 23
2.4.2 Sample size, developmental stage, and behaviours 24
2.4.3 Candidate gene 24
2.4.4 QTL mapping 25
2.4.5 GWAS 25
2.4.6 Association effect and effect of moderators 25
2.4.7 Publication bias 26
2.5 Discussion 27
2.5.1 Literature trends 27

2.5.2 Meta-analysis of literature 30
2.5.3 Challenges....32
2.5.4 Continuing role for genomics longitudinal studies, and best practices 33
2.6 References....41
Appendices....50
A.1 Additional tables 50
A.2 Additional figures 59

Chapter 3: Repeatability and reproductive consequences of boldness in female grey seals.62
3.1 Abstract....62
3.2 Introduction....62
3.3 Methods....65
3.3.1 Study site and population....65
3.3.2 Measurement of boldness scores 66
3.3.3 Measurement of reproductive success 67
3.3.4 Characterization of breeding habitat type 68
3.3.5 Factors affecting boldness....68
3.3.6 Estimating repeatability 69
3.3.7 Reproductive consequences of personality....70
3.4 Results....71
3.4.1 Boldness....71
3.4.2 Relationship between boldness and reproductive performance....72
3.5 Discussion....73
3.6 References....83
Appendices....89
B.1 Additional tables 89
B.2 Additional figures....99

Chapter 4: Genetic association with boldness and maternal performance in a free-ranging population of grey seals 100
4.1 Abstract....100

4.2 Introduction....100
4.3 Methods....105
4.3.1 Study site....105
4.3.2 Boldness measurements....105
4.3.3 Maternal performance traits....106
4.3.4 Candidate gene sequencing....107
4.3.5 Genotyping....108
4.3.6 Genotype results....109
4.3.7 Genetic diversity and neutrality tests....109
4.3.8 Association tests....109
4.4 Results....111
4.4.1 Association tests....112
4.5 Discussion....114
4.5.1 Genotype-phenotype associations....114
4.5.2 Limitations....117
4.5.3 Conclusion....118
4.6 References....128
Appendices....137
C.1 Additional tables....137
C.2 Additional figures....160

Chapter 5: Heritability and association analyses of reproductive performance in wild grey seals....170
5.1 Abstract....170
5.2 Introduction....170
5.3 Methods....174
5.3.1 Study site....174
5.3.2 Maternal trait data collection....175
5.3.3 ddRAD library preparation and post-processing....176
5.3.4 Base model selection....178
5.3.5 Quantitative genetic and genome-wide association analyses....179

5.4 Results 180
5.4.1 SNP parameter testing 180
5.4.2 Quantitative genetic estimates and GWAS 181
5.5 Discussion 182
5.5.1 Quantitative genetic assessment 182
5.5.2 Genetic architecture 184
5.5.3 Future considerations 185
5.6 References 190
Appendices 199
D.1 Double digest restriction-site associated DNA sequencing library preparation protocol 199
D.2 Additional tables 200
D.3 Additional figures 206

Chapter 6: General conclusion 208
6.1 Conclusion 208
6.2 References 213

Bibliography 216

List of Tables

Table 2.1: A list of the top ten candidate genes explored and the corresponding behavioural traits measured in association tests. 36

Table A.1: Results from various combinations of literature search term results using three databases: Web of Science, Scopus, and Google Scholar. 50

Table A.2: Categorization of behavioural trait descriptors used in summary statistics and the candidate gene meta-analysis.....52

Table A.3: List of references used in literature review according to analytical technique, with associated species, taxonomic group, and behavioural category examined (Act = Activity; Agg = Aggression; Anx/Stress = Anxiety/Stress; B = Boldness; Comm = Communication; Exp = Exploratory; For = Foraging; Hab = Habitat selection; Mig/Disp = Migratory/Dispersal; Par = Parental; Rep = Reproductive; Soc = Social). 53

Table A.4: Scatter of papers investigating the genetic architecture of natural animal behaviour published in various scientific journals between 1998 and 2018. 54

Table A.5: Full list of genes (those not included in Table 2.1) screened in the candidate gene literature with corresponding behaviours tested. With the exception of the *OXTR* gene (n = 2 studies), each gene listed was detected in only one study (n = 1 study).....55

Table A.6: Model results testing the influence of moderators on effect size across the molecular genetic literature.....57

Table A.7: Model-specific estimates of moderators investigated in assessing the influence on significant sources of effect variation. 58

Table 3.1: Parameter estimates for the preferred ordinal mixed model (clmm) assessing biological and environmental sources of variation on boldness in female grey seals on Sable Island, Nova Scotia (Canada). 78

Table 3.2: Sample sizes, mean boldness scores (± SE), and among-individual and among-year variances (95% CI) for estimation of between and within year repeatability estimates (*r*) of boldness for the Sable Island population of female grey seals. 79

Table 3.3: Parameter estimates of models generated from generalized linear mixed model (GLMM) analysis investigating the sources of variation on offspring weaning mass in grey seals (N_{obs} = 2020 on 469 females) on Sable Island, Nova Scotia (Canada). 80

Table B.1: Breakdown of study females according to cohort (year of birth) and number of observations per cohort. 89

Table B.2: Scores and descriptions of behaviours measured along the shy-bold animal personality continuum as assessed in the field (full behaviour assessment), and the collapsed assessment used for data analysis. 90

Table B.3: Developmental pelage stage of pups used when monitoring mother-pup pairs, and subsequently when assigning weaning success of females.....91

Table B.4: Category and descriptions of breeding habitat type as assessed in the field (full habitat assessment), and the collapsed habitat assessment used for data analysis.....92

Table B.5: Estimation of between year repeatability with parameter expanded priors. Variance fixed to 1 for ordinal models.....93

Table B.6: Estimation of between year repeatability with inverse gamma priors. Variance fixed to 1 for ordinal models. 94

Table B.7: Estimation of within year repeatability with parameter expanded priors. Variance fixed to 1 for ordinal models.....95

Table B.8: Top three models for assessing sources of variation on a measure of female reproductive performance, pup weaning mass, in grey seal females of Sable Island and the associated Akaike information criterion (AIC) values. Support for the top model provided by lowest AIC, smallest ΔAIC (difference in AIC from top model), and highest AIC weight (*w*)....96

Table B.9: Parameter estimates of a highly supported model investigating sources of variation on offspring weaning mass, including an interaction term between boldness and age, in grey seals (N_{obs} = 2020 on 469 females) on Sable Island, Nova Scotia (Canada). Comparisons of top three models are provided in Table B.8. 97

Table B.10: Parameter estimates of the full model from generalized linear mixed model (GLMM) analysis (N_{obs} = 2020 on 469 females) investigating sources of variation on offspring weaning mass in grey seals of Sable Island, Nova Scotia (Canada).....98

Table 4.1: Boldness scores with descriptions of behaviour, and the associated number of estimates collected for each score as well as the number of Sable Island grey seal females. Of the 180 females with repeated measures obtained from 2008-2018, the average score for shy individuals was 1.789 (SE ± 0.187; n = 95 females with 478 observations), while that of bold individuals was 4.782 (SE ± 0.0332; n = 85 females with 527 observations).....119

Table 4.2: Allele and genotype frequencies for both linked and unlinked loci detected between three candidate genes (*DRD4*, *SERT*, and *MC1R*) in 180 female grey seals of Sable Island, Nova Scotia (Canada).....120

Table 4.3: Additive model results for assessing the genetic association of three candidate genes (*DRD4, MC1R,* and *SERT*) with boldness, pup weaning mass (PWM), and lactation duration in female grey seals of Sable Island, Nova Scotia (Canada). 121

Table C.1: Total number of females behaviourally tested and listed according to the female's year of birth (cohort). 137

Table C.2: Description and number of boldness observations with respect to birth-site selection as characterized by microhabitat features. 138

Table C.3: Primers designed for targeting all or the majority of five candidate genes to examine the influence of genotype on boldness and maternal performance variation in grey seals of Sable Island, Nova Scotia (Canada). Refer to GenBank accession numbers MW864572-MW864597 and MW882063 for sequenced gene regions. 139

Table C.4: Model selection for gene-phenotype associations for boldness, pup weaning mass, and lactation duration in grey seals of Sable Island. Akaike's Information Criterion (AIC) values for AIC, ΔAIC (difference in AIC from top model), and Akaike weight (*w*) are provided. Support for the top model given by lowest AIC, smallest ΔAIC, and highest *w*.....142

Table C.5: Genetic diversity indices and Tajima's *D* neutrality test estimates for each candidate gene region sequenced. k is the average number of pairwise nucleotide differences within the population. Bolded values indicate significance at $P < 0.05$.. 144

Table C.6: Matrix of FDR-corrected P values from linkage disequilibrium analysis of *SERT* loci. 145

Table C.7: Full additive model results, including covariates age, habitat, and pup sex, from preferred models assessing gene-phenotype associations for boldness, pup weaning mass (PWM), and lactation duration in female grey seals of Sable Island. Female identity and year were included in models as random factors. 146

Table C.8: Estimates of the proportion of trait variation [boldness, pup weaning mass (PWM), and lactation duration (LD)] explained by each gene, SNP, and other fixed factors (i.e. age, habitat, and pup sex), as well as all loci combined, calculated from marginal R^2 ($R^2_{GLMM(m)}$).

Female identity and year were included in models as random factors. Conditional R^2 ($R^2_{GLMM(c)}$) also provided. 149

Table C.9: Overdominance (OD) model results for the genetic effect on boldness in female grey seals of Sable Island, Nova Scotia (Canada). Results are based on a sample size of up to 180 females and 1183 observations over an 11-year period. Female identity and year were included in models as random factors. 151

Table C.10: Overdominance (OD) model results for the genetic effect on pup weaning mass in female grey seals of Sable Island, Nova Scotia (Canada). Results are based on a sample size of up to 177 females and 1496 observations over a 28-year period. Female identity and year were included in models as random factors. 153

Table C.11: Overdominance (OD) model results for the genetic effect on lactation duration in female grey seals of Sable Island, Nova Scotia (Canada). Results are based on a sample size of up to 151 females and 542 observations over a 28-year period. Female identity and year were included in models as random factors. 155

Table C.12: Additive model results using alternate loci from *SERT* linkage group (loci approaching significance and linked with *SERT*_5987). Alternate loci demonstrated similar effect sizes to those presented for association between *SERT*_5987 and boldness, pup weaning mass (PWM), and lactation duration. Female identity and year were included in models as random factors. 157

Table 5.1: Animal models used to estimate additive genetic variance and heritability for each maternal trait measured in grey seals of Sable Island, Nova Scotia (Canada). 187

Table 5.2: Number of individuals and observations, range, mean, repeatability, heritability, and genomic inflation factors for eight traits measured in female grey seals of Sable Island, Nova Scotia (Canada). 188

Table D.1: Breakdown of 485 grey seal females used for genomic analyses according to year of birth (cohort). 200

Table D.2: Descriptions of grey seal pup developmental stages used to assess female reproductive status and birth date for stage 1 pups on Sable Island, Nova Scotia (Canada).....201

Table D.3: Behavioural scores of grey seal females measured along the shy-bold continuum on Sable Island, Nova Scotia (Canada) from 2008-2019. 202

Table D.4: Comparisons of top models to assess sources of variation on each female grey seal trait and associated AIC (Akaike Information Criterion) values. Support for the top model was provided by the lowest AIC, smallest ΔAIC (difference in AIC from top model), and highest AIC weight (*w*), while taking into account missing phenotypic data for putative fixed factors. 203

Table D.5: Repeatability estimates for morphological, life-history, and boldness traits in Sable Island female grey seals and associated model structures used for analyses.....205

List of Figures

Figure 2.1: Bar graph showing the number of articles using candidate gene, QTL mapping, and GWAS approaches to study the genetic basis of behaviour in natural systems over time. 37

Figure 2.2: Bar graph showing a taxonomic breakdown of the number of studies published using each analytical technique to study the genetic basis of behaviour in natural systems.....38

Figure 2.3: Forest plot of average effect size estimates (± 95% CIs) for each moderator tested to assess the magnitude of effect in association and linkage analyses of molecular behavioural studies. 39

Figure 2.4: Funnel plot for the detection of publication bias in the literature assessing the genetic basis of behaviour in natural, or semi-natural, populations. 40

Figure A.1: Sample size of subjects for each molecular analytical method used to explore the genetic basis of behavioural phenotypes.....59

Figure A.2: Comparison of the number of markers used between quantitative trait locus (QTL) mapping and genome wide association study (GWAS) approaches. 60

Figure A3: Phylogenetic tree and associated forest plot illustrating average effect size for species analyzed in studies assessing the molecular genetic basis of behaviour in natural, or nearly natural, populations. 61

Figure 3.1: The mean boldness scores according to maternal age and habitat type of female grey seals breeding at the Sable Island, Nova Scotia (Canada) gray seal colony. 81

Figure 3.2: The relationship between maternal age and boldness with pup weaning mass (measured to the nearest 0.5 kg) in the Sable Island population of female gray seals. 82

Figure B.1: The relationship between maternal age and boldness with pup weaning mass (measured to the nearest 0.5 kg) in the Sable Island population of female grey seals. The mean weaning mass for each age bracket measured along a shy-bold continuum is plotted with ± standard error. 99

Figure 4.1: Map showing the location of Sable Island National Park Reserve of Nova Scotia, Canada where female grey seals have been monitored annually since the 1980s and behaviourally assayed from 2008-2018. 123

Figure 4.2: Schematics of gene structure for five candidate genes [serotonin transporter (*SERT*), dopamine receptor D4 (*DRD4*), melanocortin receptors 1 and 5 (*MC1R* and *MC5R*), and oxytocin receptor (*OXTR*)] used to assess the relationship between genotype and boldness and maternal performance variation in female grey seals of Sable Island, Nova Scotia (Canada). ...124

Figure 4.3: Boldness of female grey seals and the additive allele effect of the serotonin transporter (*SERT*) gene. Boldness showed associations with genotypes at: A) the *SERT*_15636 locus; and, B) the *SERT*_12472 locus. Sample size of females used for analyses are included above each genotype and number of behavioural observations are above the genotype mean. Error bars represent standard error of the genotype mean. The corresponding test statistics are provided in Table 4.3. 125

Figure 4.4: Relationship between the additive allele effect and two measures of grey seal maternal performance, pup weaning mass (PWM) (A and B) and lactation duration (C and D), at loci of two candidate genes that showed trends with each performance trait. Sample size of females used for analyses are included above each genotype and number of behavioural observations are above the genotype mean. Error bars represent standard error of the genotype mean. The corresponding test statistics are provided in Table 4.3. 126

Figure 4.5: Forest plots of effect size estimates (± 95% confidence intervals) for the association of seven loci across three candidate genes with boldness, pup weaning mass, and lactation

duration in female grey seals of Sable Island, Nova Scotia (Canada). Size of the point is proportional to the number of observations used in association tests.....127

Figure C.1: Plots of residuals to ensure selection of females was appropriate for study objectives. A GLM was performed correcting for age and habitat on boldness using the whole dataset, and subsequently estimated the mean residual for each female and plotted that against the mean raw boldness score (left), confirming selection of females was appropriate. Blue dots represent seals included in the current study and grey dots of females not included. Also plotted are the mean residual values (right). There are instances where females falling into the 'shyest' and 'boldest' regions were not included in the study; however, genetic samples were not available for all females for which boldness scores were obtained. 160

Figure C.2: Principal component analysis (PCA) plot indicating no significant population structure within the Sable Island population of female grey seals of our study. The PCA analysis was completed with missing data imputed on a small number of loci (N = 7) using the R package 'missMDA' (Josse and Husson 2016) and the PCA was completed using the 'prcomp' function. The imputation of missing data on this small dataset will potentially result in the appearance of clusters. In addition, these loci are distributed on three different genes, and have non-statistically significant levels of linkage. In datasets with no population structure, the appearance of linkage will result in strong clustering of individuals (e.g., Figure 2a in Trevoy et al. 2019 and Figure 3 in Lumley et al. 2020). 161

Figure C.3: Boxplot showing the relationships between boldness and two measures of maternal performance, pup weaning mass (PWM) and lactation duration. Horizontal line within the box represents the median values for maternal performance traits measured. In the weaning mass panel, the median value of PWM for bold females was 54 kg (n = 606 observations; range is 22.5-77 kg) and that of shy females was 53 kg (n = 353 observations; range is 27-72 kg). In the lactation duration panel, the median was 18 days (n = 123 observations; range is 9-22 days) for bold females and 17.5 days (n = 98 observations; range is 9-22 days) for shy females.....162

Figure C.4: The relationships between boldness and two measures of maternal performance, pup weaning mass (PWM) and lactation duration, in a subset of Sable Island grey seal females (n = 188) having either extreme shy (scores 1 and 2) or bold (scores 4-6) phenotypes, with repeated measures collected of boldness collected over an 11-year period. A boldness score of 3 (intermediate, no boldness) could be a reflection of shy or bold females in certain years of data collection. 163

Figure C.5: Boldness scores for female grey seals in relation to SNP genotypes for seven unlinked loci identified across three candidate genes (*MC1R*, *DRD4*, and *SERT*). The number of females and repeated observations used for analyses is provided above each genotype and the genotype mean value, respectively. Error bars represent standard error of the genotype mean. The corresponding test statistics are provided in Table 4.3 of the manuscript. 164

Figure C.6: Pup weaning mass (PWM) values, a reproductive performance parameter for female grey seals, in relation to SNP genotypes for seven unlinked loci identified across three candidate genes (*MC1R*, *DRD4*, and *SERT*). The number of females and repeated observations used for analyses is provided above each genotype and the genotype mean value, respectively. Error bars represent standard error of the genotype mean. The corresponding test statistics are provided in Table 4.3 of the manuscript. 165

Figure C.7: Lactation duration of female grey seals in relation to SNP genotypes for seven unlinked loci identified across three candidate genes (*MC1R*, *DRD4*, and *SERT*). The number of females and repeated observations used for analyses is provided above each genotype and the genotype mean value, respectively. Error bars represent standard error of the genotype mean. The corresponding test statistics are provided in Table 4.3 of the manuscript. 166

Figure C.8: Boldness scores for female grey seals in relation to SNP genotypes for three loci whose minor allele homozygote genotypes were rare in the population. Grouping the minor allele (allele grouped) and eliminating the rare genotype from analyses (genotype eliminated) was done to ensure that rare genotypes were not disproportionately influencing the relationship between genotype and boldness. The number of females and repeated observations used for analyses is

provided above each genotype and the genotype mean value, respectively. Error bars represent standard error of the genotype mean.....167

Figure C.9: Average pup weaning mass (PWM) scores for female grey seals in relation to SNP genotypes for three loci whose minor allele homozygote genotypes were rare in the population. Grouping the minor allele (allele grouped) and eliminating the rare genotype from analyses (genotype eliminated) was done to ensure that rare genotypes were not disproportionately influencing the relationship between genotype and PWM. The number of females and repeated observations used for analyses is provided above each genotype and the genotype mean value, respectively. Error bars represent standard error of the genotype mean.....168

Figure C.10: Average lactation duration (LD) scores for female grey seals in relation to SNP genotypes for three loci whose minor allele homozygote genotypes were rare in the population. Grouping the minor allele (allele grouped) and eliminating the rare genotype from analyses (genotype eliminated) was done to ensure that rare genotypes were not disproportionately influencing the relationship between genotype and LD. The number of females and repeated observations used for analyses is provided above each genotype and the genotype mean value, respectively. Error bars represent standard error of the genotype mean.....169

Figure 5.1: Manhattan plots for associations between SNPs and eight female grey seal traits: length at primiparity, age at primiparity, pup birth mass, lactation duration, birth day, boldness, maternal mass, and pup weaning mass. The red dashed line indicates a significance threshold using Bonferroni correction for the number of loci used (α = 0.05/number of loci).....189

Figure D.1: Comparisons of effects from varying Stacks parameters *M*, *m,* and *n* on the number of assembled loci, polymorphic loci, and SNPs for 30 grey seal females of Sable Island. 206

Figure D.2: Reduced major axis regression results depicting the relationship between significant repeatability and heritability estimates for repeat measured traits (lactation duration pup weaning mass, maternal mass, birth date, and boldness) of female grey seals from Sable Island.....207

Chapter 1: General Introduction

1.1 Introduction

1.1.1 Individual variation

No one supposes that all individuals of the same species are cast in the same mould (Darwin, *On the Origin of Species*, 1859). Individual variation in phenotypic traits serves as material upon which natural selection acts, leading to adaptation and evolutionary change. This among-individual phenotypic variation is a natural feature found ubiquitously, and when linked with fitness, has important consequences for population growth rates and viability. Life histories (e.g., age and size at primiparity, growth rates, reproductive investment, and mortality), for instance, are key determinants of lifetime reproductive success, and as such, are firmly planted at the interface between ecology and evolution (Stearns 1992). Where phenotypic differences exist among individuals in a population, such as those following different reproductive schedules, foraging strategies, and risk-taking behaviour, there will be individuals having particular phenotypes that confer higher survivability and reproductive success than that of their counterparts possessing 'lesser' phenotypes (Forsythe et al. 2021). This individual variation influences demographic outputs and population vital rates, shaping ecological processes and trait distributions across time within free-living populations (Stearns 1992). A primary goal in evolutionary biology is to provide insight into the nature of phenotypic variation, from its source and maintenance in the wild to the consequences of such variation on fitness. With exposure to environmental and novel anthropogenic pressures (Allendorf and Hard 2009; Steffen et al. 2011), understanding variation and predicting the evolutionary potential of traits as well as the rate of adaptation in response to changing conditions is fundamental (Pelletier and Coltman 2018).

1.1.2 Behavioural variation and animal personality

Biologists have long noted the existence of individual differences in behaviour in their study systems, as evident by popular books such as *Wild Wolves We Have Known* (Thiel 2013) and *In the Shadow of Man* (Goodall 1971). These stories highlight certain individual animals that routinely demonstrated particular behavioural characteristics different from their conspecifics. Behaviour determines how an individual responds to and interacts with its environment, and as such is recognized as a crucial, albeit rarely considered, component of wildlife management and

conservation planning (Caro 2007). Within the field of behavioural ecology, animal personality research represents one of the most rapidly growing areas of study (Carere and Maestripieri 2013). Animal personality is defined as consistent individual differences in behaviour across time and contexts (Gosling 2001; Réale et al. 2007). Moving beyond field anecdotes and a view that individual variation in behaviour is merely statistical noise around an adaptive or "golden" mean (Fox et al. 2009), animal personality research has revealed much on how inter-individual differences in behaviour affect various ecological processes. For instance, animal personality trait variation has been linked with inter- and intraspecific interactions, reproductive success, foraging strategies, and habitat and other resource use – associations that directly affect population productivity and persistence (Wolf and Weissing 2012). Furthermore, animal personality trait variation is subject to natural and sexual selection, and suites of correlated traits (i.e., behavioural syndromes) readily evolve (Dingemanse and Réale 2005). As this behavioural variation is found throughout the animal kingdom, documented in species from field crickets (*Gryllus campestris*) (Niemelä et al. 2015) to spotted hyenas (*Crocuta crocuta*) (Yoshida et al. 2016), animal personality represents an exciting avenue of research within the framework of evolutionary biology and life-history theory (Carere and Maestripieri 2013).

1.1.3 Genetic basis of phenotypic variation

Determining the genetic basis of traits can provide insight into important eco-evolutionary processes, such as those driving the maintenance of variation in natural populations (Santure and Garant 2018), as well as of the adaptive and evolutionary potential of traits (e.g., Rivrud et al. 2019 and Duntsch et al. 2020). Estimating repeatability, a population parameter that describes the proportion of phenotypic variance that is due to individual differences (Falconer and Mackay 1996), is often a first step taken when examining the genetic basis of a trait. Repeatability can set the upper bounds to heritability (i.e., phenotypic variation that is due to genetic differences between individuals) by taking into account both genetic and environmental sources of trait variation (but see Dohm 2002), and further represents a measure to determine inter-individual consistency in a trait (Boake 1989; Bell et al. 2009; Stamps and Groothuis 2010; Biro and Stamps 2015).

Broad-sense heritability (H^2) describes how much variance in a trait is due to total genetic variance ($H^2 = V_G/V_P$), whereas heritability in the narrow-sense (h^2) is defined as the proportion

of phenotypic variance that is attributable to additive genetic variance ($h^2 = V_A/V_P$) (Falconer and Mackay 1996). Narrow-sense heritability is the commonly used measure to determine whether genetic variation underlying a trait exists (Hoffmann et al. 2017), providing inference of the adaptive potential and rate of response to selection. Because heritability relies on knowledge of genomic relatedness between individuals in a population, many studies have been predominantly limited to few natural systems with existing pedigree data (Postma 2014).

In addition to repeatability and heritability of traits, determination of a trait's genetic/genomic architecture (e.g., number of loci, genomic distribution of loci, and magnitude of loci effect) has been an important area of research in exploring the genetic basis of traits (Stinchcombe and Hoekstra 2008; Santure and Garant 2018). Techniques such as candidate gene association tests, quantitative trait locus (QTL) mapping, and genome-wide association studies have been readily used in an effort to better understand the effects of genetics on trait variation in addition to identifying the genes or genomic regions favored by selection within populations (van Oers et al. 2010). Until recently, investigating the genetic architecture of traits has been limited to studies on model, laboratory, and domesticated species, with relatively few studies performed using natural or semi-natural systems (Stapley et al. 2010). However, the advent of novel molecular tools (i.e., high-throughput sequencing technologies) has radically improved accessibility, extending research to many species of free-living populations (Santure and Garant 2018).

1.1.4 DNA sequencing technologies

Advances in sequencing technologies have made it possible to rapidly sequence hundreds to thousands of genetic markers within and between populations in practically any species (Andrews et al. 2016). Prior to these advances in high-throughput sequencing methods, researchers were restricted by a limited number of loci (e.g., mitochondrial DNA and microsatellites) with which to type individuals and to investigate patterns of genomic variation (Luikart et al. 2003; Allendorf et al. 2012), and some research was further limited to model organisms or species with which genomic resources were available. Concurrent with increased accessibility and decreasing costs, next-generation sequencing techniques have contributed much to our understanding of the evolutionary biology and ecology of many free-living populations. Representative reference genomes now exist for, at minimum, a closely related species in many

taxonomic groups (Allendorf et al. 2010; Cammen et al. 2016), facilitating the opportunity to apply genomic methods to a study system of interest in an effort to address previously resolute research questions. Further facilitating research of wild populations are reduced-representation library (RRL) sequencing methods that are used to evaluate only a subset of markers randomly distributed throughout the genome. Rather than sequencing whole genomes, RRL sequencing approaches permit inclusion of a larger number of individuals to genotype at thousands of markers in a cost-effective manner (Davey et al. 2011). A popular RRL sequencing method is the restriction site-associated DNA sequencing (RADseq) technique (Cammen et al. 2016). RADseq methods use one or more restriction enzymes to produce sequence data that simultaneously identifies and genotypes thousands of markers throughout the genome (Baird et al. 2008; Peterson et al. 2012; Andrews et al. 2016). The utility of RADseq in studies with a large number of individuals makes it an attractive molecular method to generate genomic data for examining the genetic basis of traits in the wild.

1.1.5 Marine mammals

Marine mammals represent a unique group of animals with which to investigate the evolutionary dynamics and adaptive potential of behavioural variation as well as other quantitative, fitness-related traits (Cammen et al. 2016). These animals have undergone multiple and independent evolutionary transitions (McGowen et al. 2014), evolving exceptional morphological, physiological, and behavioural adaptations. Following years of persecution and unsustainable harvesting for their blubber, meat, bones, and fur, many marine mammal populations are still recovering, some of which remain critically endangered. Marine mammals play a vital role in healthy marine ecosystems, from serving as apex predators to dispersing nutrients from areas of high to low productivity (Bowen 1997; Roman et al. 2014; Kiszka et al. 2015). Nevertheless, studying individual variation in marine mammals does not come without its unique set of logistical challenges and difficulties, such as those associated with the proportion of time spent underwater by marine mammals, their long lifespans, marking and/or identifying individuals, and the high costs associated with marine research (Bowen 1997). These challenges may contribute to why studies on individual variation of marine mammals are relatively limited and are focused predominantly on captive animals. The amphibious nature of pinnipeds (i.e.,

seals, sea lions, and walruses), however, presents an opportunity to assess individual variation in quantitative traits when hauled out on land for extended periods of time to breed, pup, and molt.

1.1.6 Grey seals

The grey seal (*Halichoerus grypus*), member of the Phocidae or 'true seal' family, is an iteroparous and long-lived species, with males and females living into their 30s and 40s, respectively. Grey seals are sexually size-dimorphic, such that males (up to 350 kg) are approximately 1.5 times larger than females (up to 250 kg) (Beck 2002; DFO 2014). They are a philopatric and colonial species, hauling out on land or ice annually to give birth and to mate (Mansfield and Beck 1977), and are comprised globally of three main breeding stocks: Northwest Atlantic, Northeast Atlantic, and Baltic Sea. Beginning around 4 to 6 years of age, females birth a single pup on a near annual basis and provide all parental care during a brief lactation period that lasts on average 16 to 18 days (Boness and James 1979; Iverson et al. 1993). During this time, a female must transfer enough milk energy to her pup to bring it to a condition conducive for early-life survival until it reaches foraging independence (approximately 3-week postweaning fast) (Iverson et al. 1993; Mellish et al. 1999; Noren et al. 2008). As capital breeding strategists (Boness and James 1979), female grey seals fast for the duration of time spent while hauled out during the pupping and breeding season, and thus rely on stored energy reserves for nursing and sustaining her own metabolic needs (Iverson et al. 1993). While a female loses approximately a third of her body mass during the 16- to 18-day window (Mellish et al. 1999), her pup can more than triple its body mass owing to the fat rich milk that she delivers (Bowen et al. 1992; Iverson et al. 1993). In addition to supplying milk, females must physically protect her offspring from aggressive conspecifics, land predators, and other threats (Kovacs 1987). Much variation in the quality of maternal performance exists among grey seals, with consistent individual differences established over time for various fitness-related traits, such as daily milk output, pup weaning mass, and pup-checking rates, across breeding colonies (Lang et al. 2009; Twiss et al. 2012). This suggests that selection for individual differences in grey seals is strong and creates an impetus to examine the adaptive potential of these traits as well as to explore the proximate and ultimate mechanisms generating and maintaining variation.

1.1.7 Longitudinal studies: Sable Island

Longitudinal field programs spanning multiple decades are invaluable to ecological research (Clutton-Brock and Sheldon 2010a), providing not only the opportunity to assess trends within a population over time but also for the individual-based records that are often maintained for hundreds to thousands of animals with which to explore mechanisms of individual variation. Sable Island National Park Reserve in Nova Scotia, Canada has been the focus of a long-term monitoring effort on grey seals by Canada's Department of Fisheries and Oceans (DFO). Beginning in the 1960s, DFO has extensively monitored and documented the recovery of Sable Island grey seals. Sable Island experienced an annual pup production rate of 13% from 1976 to 1997 (Bowen et al. 2003, 2007), with slowed growth (5-7%) observed beginning in the mid-2000s and continuing today as the population likely approaches carrying capacity. The island currently supports the world's largest breeding colony of grey seals, wherein recent estimates suggest that roughly 370,000 seals haul out (Hammill et al. 2017) and produce over 87,000 pups annually (80% of total pup production in the Northwest Atlantic stock, den Heyer et al. 2021). DFO has permanently marked a subset of the grey seal population at Sable Island. Data on branded individuals have permitted, among other monitoring and research objectives, assessments of population recruitment (e.g., Bowen et al. 2015), demographics (e.g., den Heyer et al. 2021), and individual reproductive performance (e.g., Badger et al. 2020). Life-history profiles for many branded females are known and include data such as age at first parturition, birth date, pup weaning mass, and lactation duration. This extensive life-history dataset, in addition to archived tissue samples, permits investigating the genetic basis of behaviour and reproductive performance traits in female grey seals of Sable Island.

1.2 Thesis objectives and data chapters

The overall objective of my doctoral research is to investigate the genetic basis of maternal performance variation in female grey seals in an effort to provide insight into the adaptive potential of traits in the Sable Island population as well as to further our understanding of the nature of variation in fitness-related traits. This thesis includes four data chapters wherein I: 1) examine analytical molecular approaches that are commonly and contemporaneously used to elucidate the genetic basis of animal behaviour in natural populations, 2) estimate the repeatability of behavioural trait variation along the shy-bold continuum and describe the

relationship between boldness and offspring weaning mass in female grey seals of Sable Island, 3) use a candidate gene approach to test for association of genetic markers with three maternal performance traits, and 4) perform a RRL sequencing method and use the genome-wide single nucleotide polymorphisms (SNPs) identified to estimate heritability and assess the genetic architectures of morphological, life-history, and behavioural traits in Sable Island grey seals.

In **Chapter 2**, I perform a literature review and meta-analysis to explore recent trends in analytical approaches used to investigate the relationship between genes and behaviour in natural systems, specifically candidate gene approaches, QTL mapping, and genome-wide association studies. I evaluate and discuss the usefulness and reported successes of each approach, while also describing which behaviours and species are examined by researchers most often. I further quantify the magnitude of effect for each analytical approach taken by study authors, as well as of the effect for source of population, taxa, and behaviour assayed.

In **Chapter 3**, I examine the repeatability of boldness, a trait that effectively assesses maternal defense of offspring, using repeated behavioural measures collected for 469 female grey seals over a nine-year period on Sable Island. I investigate the relationships between environmental and biological factors with boldness variation, and further determine the effect of boldness on an important predictor of maternal performance and reproductive success in the grey seal, offspring weaning mass.

In **Chapter 4**, I use a candidate gene approach to investigate the association of genetic variants with repeated measures of boldness, offspring weaning mass, and lactation duration collected over multiple years as part of the Sable Island longitudinal field program. I isolate and re-sequence five candidate genes, dopamine receptor D4, serotonin transporter, oxytocin receptor, and melanocortin receptors 1 and 5, that have previously been linked with behavioural variation in other organisms. With the discovered SNPs in these genes, I test for genotype-phenotype relationships in a reduced dataset of 180 females having extreme shy-bold phenotypic values.

In **Chapter 5**, I examine the quantitative genetics and underlying genetic architectures of morphological, life-history, and behavioural traits in female grey seals of Sable Island. Using a RADseq approach, I generate a panel of genome-wide SNPs for nearly 500 female grey seals. With this SNP data, I create a genomic relatedness matrix to estimate narrow-sense heritability

values of eight maternal traits. Additionally, I perform genome-wide association analyses using existing phenotypic data from the Sable Island longitudinal field program.

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Chapter 2: The genetic basis of animal behavioural diversity in natural populations

2.1 Abstract

Individual differences in animal behaviour influence ecological and evolutionary processes. Much behavioural variation has a heritable component, suggesting that genetics may play a role in its development. Yet, the study of the mechanistic description linking genes to behaviour in nature remains in its infancy, and such research is considered a challenge in contemporary biology. Here, we performed a literature review and meta-analysis to assess trends in analytical approaches used to investigate the relationship between genes and behaviour in natural systems, specifically candidate gene approaches, quantitative trait locus (QTL) mapping, and genome-wide association studies (GWAS). We aimed to determine the efficacy and success of each approach, while also describing which behaviours and species were examined by researchers most often. We found that the majority of QTL mapping and GWAS results revealed a significant or suggestive effect [Zr = 0.3 (95% CI: 0.25:0.35) and Zr = 0.39 (0.33:0.46), respectively] between the trait of interest and genetic marker(s) tested, while over half of candidate gene accounts [Zr = 0.16 (0.11:0.21)] did not find a significant association. Approximately a third of all study estimates investigated animal personality traits; though, reproductive and migratory behaviours were also well-represented. Our findings show that despite widespread accessibility of molecular approaches given current sequencing technologies, efforts to elucidate the genetic basis of behaviour in free-ranging systems has been limited to relatively few species. We discuss challenges encountered by researchers, and recommend integration of novel genomic methods with longitudinal studies to usher in the next wave of behavioural genomic research.

2.2 Introduction

Behaviour dictates how an animal interacts with its environment, and as such, is tightly linked with an individual's lifetime reproductive success and survival. Researchers have begun paying increased attention towards the study of interindividual behavioural diversity within a

population or species, and moreover towards the study of animal personality (Carere and Maestripieri 2013). Consistent individual differences in behaviour influence natural selection by affecting various ecological processes, including life-history characteristics, habitat selection, and responses to changing environmental conditions and anthropogenic pressures (reviewed in Wolf and Weissing 2012). Consequently, behavioural variation directly influences how populations adaptively evolve. Why this variation persists is difficult to explain, however, as natural selection is expected to erode variation leading to an adaptive population mean (Taylor and Williams 1982; Tomkins et al. 2004). While hypotheses, such as balancing selection (Turelli and Barton 2004), life-history trade-offs (Wolf et al. 2007), and frequency-dependent mechanisms (Dall et al. 2004), provide some support for the generation and maintenance of behavioural variation, 'why and how is variation maintained?' remain fundamental questions in evolutionary biology. It is thus the continuing interest and consideration of proximate causes, such as genetics, and the ultimate mechanisms underlying individual behavioural variation that is critical in addressing these evolutionary questions.

Many quantitative genetic studies of animal behaviour and personality have revealed moderate to high estimates of heritability, ranging from 20%-50% (Dochtermann et al. 2019; Dochtermann et al. 2015; Postma 2014; Stirling et al. 2002; van Oers and Sinn 2013), suggesting that behavioural diversity may be encoded for in the genome. Yet, analysis of the genetic architecture (e.g., number of loci involved, genomic distribution, magnitude of effects, and allelic relationships) of behavioural traits remains largely underexplored compared to that of developmental and life-history characteristics (Boake et al. 2002). Understanding the structure and function of the genetic architecture underlying complex traits such as behaviour may provide insight into how phenotypic traits respond to selection, influencing population adaptation and future evolutionary dynamics (Clutton-Brock and Sheldon 2010a; Laine and van Oers 2017). Like other quantitative traits, behaviour is thought to be governed by many genes of small effect (i.e. polygenic) (Laine and van Oers 2017; Santure et al. 2015), many of which may be in linkage disequilibrium (LD) or have epistatic interactions. As a result, selection may proceed more slowly than if traits were controlled by a few genes of large effect (i.e. oligogenic) (Hill and Robertson 1966). As each quantitative trait locus (QTL) detected is expected to explain only a small part of the total behavioural variance observed (Flint 2003), and because it is further unclear to what extent factors such as nonadditive genetic effects and the environment have in

shaping behaviour, studying the genetic basis of behavioural variation in free-ranging populations has been referred to as one of the greatest challenges in biology today (Chakarov et al. 2013).

Most information currently available on the genetic mechanisms underlying behavioural traits comes from research on humans, laboratory model organisms, and domesticated animals bred for production (Flint 2003; van Oers and Sinn 2013). However, repeated breeding in captivity and domestication often results in shifts of animal behaviour (McDougall et al. 2006), and can lead to less variation in behaviour than is observed in natural settings (Laine and van Oers 2017). For example, domesticated animals may exhibit reduced reactivity during behavioural assays, such as those designed to assess aggressiveness and boldness, as these animals are selectively bred to be adapted to their environments (Mormède 2005). In addition, selection caused by laboratory environments may alter genetic variance relative to those of wild populations (Kruuk et al. 2008). Therefore, studies of laboratory or domesticated animal systems may not reflect standing natural behavioural and genetic diversity, and may further fail to shed light on processes shaping and maintaining interindividual behavioural variation in nature (Crusio 2015). Fortunately, methodological advances in genomic technologies put genetic resources and tools within reach for nearly any species, allowing for the identification of genetic markers with which to study the molecular basis of behavioural diversity (Ellegren 2014). Reviewed extensively elsewhere (e.g., Bell 2008; Bendesky and Bargmann 2011; Boake et al. 2002; van Oers and Mueller 2010), three analytical strategies have repeatedly been discussed as plausible avenues for investigating the genetic basis of behavioural diversity: candidate gene, QTL mapping, and genome-wide association study (GWAS) methods.

A candidate gene association test is a hypothesis-driven approach, wherein a gene of known function or one that is suspected to influence the expression of a particular trait is screened to assess the relationship between genetic polymorphisms and phenotypic variants. Typically identified in model organisms, candidate genes are subsequently tested in species of interest to determine if genetic variants influence a similar phenotype (Fitzpatrick et al. 2005). Genes selected for testing are often those that have a major effect on a phenotypic trait (Barrett and Hoekstra 2011), and are located by researchers via a search of the relevant literature, genome databases, and/or are known to reside in a particular biological pathway (Fitzpatrick et al. 2005). This approach is an attractive option for species with which genomic resources and relatedness

information may not be available. As with other associated-based methods, a correlative relationship between genotype and phenotype does not guarantee a functional link, as the marker analyzed could be in linkage with the causal variant (Hartl and Clark 2007). Nevertheless, a candidate gene approach serves as a good starting point and/or complementary approach to other analytical techniques (Laine and van Oers 2017; van Oers and Mueller 2010).

Unlike candidate gene association studies, the QTL mapping technique is a bottom-up approach that offers a genome-wide perspective, requiring no *a priori* gene selection information (van Oers and Mueller 2010; Slate et al. 2010). QTL mapping explores linkage between genome-wide genetic markers and phenotypic variation in populations segregating for a trait (Lynch and Walsh 1998; Slate 2005). Requiring detailed pedigree information or controlled crosses between two closely-related populations or strains (Lynch and Walsh 1998), QTL mapping is an approach with limited utility in some species. Yet, much has been learned from QTL mapping of production traits related to behaviour in domesticated animals (Mormède 2005). For instance, in a large F2-population of domesticated silver foxes (*Vulpes vulpes*), Nelson et al. (2017) discovered eight loci, and three pairs of epistatic loci, associated with the regulation of aggression and tameness. Reports such as these are encouraging, but it is less clear to what extent QTL mapping studies have contributed to our understanding of the genetic mechanisms underlying behavioural traits in wild or semi-wild populations.

A GWAS is an analytical technique used to detect associations between trait(s) of interest and novel genetic variants in groups of unrelated individuals (see Visscher et al. 2017); hence, GWAS analyses can be performed on organisms with which prior genomic resources and pedigree information are not available. Performing a GWAS requires the use of a high-density panel of genetic markers, often many thousands of single nucleotide polymorphisms (SNPs), permitting locating QTL in genomic regions of relatively small effect size (Mackay et al. 2009). While both QTL mapping and GWAS are performed using genetic markers with no *a priori* connection to the traits examined, QTL mapping is dependent upon physical linkage between the marker and causal loci that segregate together in a pedigree, whereas GWAS relies on linkage disequilibrium between the marker and trait of interest in a population. Development of next-generation sequencing technologies, concurrent with decreasing costs and widespread accessibility, now facilitates the use of this analytical approach in non-model study systems of natural populations (Baird et al. 2008; Ellegren 2014; Miller et al. 2007). Some studies of wild

populations have successfully identified loci influencing particular traits, notably morphological and life-history characteristics, using a GWAS approach. Husby et al. (2015) detected a significant SNP that explained 3.9% of clutch size variance in a wild population of collared fly-catchers (*Ficedula albicollis*). Similarly, loci influencing horn type and leg length phenotypes in free-ranging Soay sheep (*Ovis aries*) have been detected using the GWAS approach (Johnston et al. 2011 and Bérénos et al. 2015, respectively). Less is known of GWAS findings for variation in behavioural phenotypes, especially in natural systems.

Laine and van Oers (2017) recently provided an overview of current progress in the study of animal personality and behavioural diversity using quantitative and molecular genetic approaches. The authors highlight the function and significance of the aforementioned molecular approaches, and in doing so, feature frequently tested candidate genes, discuss the success of QTL mapping attempts to date, and the general utility of GWASs. While their aim was to focus on studies from wild populations, many examples reported came from studies on humans, rodents, and livestock (Laine and van Oers 2017). Therefore, it remains to be seen if the call for molecular genetic and genomic studies of animal behaviour and personality traits in natural populations has been met with empirical research (van Oers and Sinn 2013). Here, we extend the work of Laine and van Oers (2017) by exploring recent trends in research methodologies, and the efficacy of those methodologies in elucidating the genetic basis of interindividual behavioural differences in natural systems. We further ask: a) Has there been success and advancement in our understanding of the molecular genetic basis of behavioural traits in natural populations; b) are certain taxa, or species, dominating research in the field; and c) have particular behaviours been met with more research? To address these objectives, we performed a comprehensive literature review and meta-analysis of empirical studies that used either a candidate gene association test, QTL mapping, and/or a GWAS approach in the examination of the genetic basis of behaviour in natural, or semi-natural, populations.

2.3 Methods

2.3.1 Literature search and inclusion criteria

We used review guidelines as outlined by Pullin and Stewart (2006) to examine the literature on the molecular genetic basis of animal behavioural diversity. To perform our review, we searched for published studies using inclusive search terms in the Scopus, Web of Science,

and Google Scholar databases between June 25 and July 6, 2018. We searched for such studies with various combinations of the following search terms: animal behav*, personality, behav* syndrome, coping style, temperament, candidate gene, QTL mapping, QTL analysis, genome-wide association, and GWAS. For instance, the Boolean operator "personality" AND "candidate gene" was used when searching for studies investigating the genetic basis of personality using a candidate gene approach (refer to Table A.1 for search term results). In addition to using the aforementioned search engines, we reviewed the reference list of each paper to include references not identified in the initial primary search.

Studies retained for analysis investigated the genetic basis of behavioural variation in wild populations, semi-wild populations (i.e. wild caught and hand-reared), or captive individuals descended from recently collected non-domesticated animals. We chose not to include studies on domesticated animals and model organisms bred in captivity for laboratory experiments in an effort to assess the mechanistic basis of genetic and behavioural variation in natural, or nearly natural, systems. We did include studies that examined animals in zoo settings, as well as those of ecological model systems, such as honey bees (*Apis mellifera*), zebrafish (*Danio rerio*), and great tits (*Parus major*). We further included studies of classically defined model organisms if individuals were wild-caught or if they had been descended from individuals recently sampled from the wild. For instance, Krackow and König (2008) used offspring of wild-caught house mice (*Mus musculus domesticus*) as study animals with which to investigate the genetic basis of a dispersal-related trait.

Only full-account papers were included in an effort to extract all relevant information pertaining to our study objectives, and as such, published abstracts and conference proceedings were excluded. We also excluded review papers and chapters focused on individual species or taxa and on particular candidate genes. For candidate gene studies, we did not include studies that solely investigated patterns of gene expression, as we aimed to focus our review on the direct evaluation of genetic polymorphisms (i.e. allelic variation) affecting behavioural differences. Lastly, only studies explicitly testing a behavioural trait, and not of those that assessed a morphological trait previously hypothesized to be associated with behavioural variation among individuals, were included. While life-history traits have previously been excluded from meta-analyses on components of behaviour (e.g., Bell et al. 2009 and Dochtermann et al. 2019), we included such traits, specifically reproductive timing (e.g., timing

of breeding, laying date, and arrival date), as part of our data collection if traits were categorized by researchers as behaviourally-based (e.g., Chakarov et al. 2013 and Krawczak et al. 2005).

In instances where papers examined the genetic basis of behaviour in more than one species, we considered each analysis as a separate entry. Few papers used more than one technique (e.g., GWAS and QTL mapping or candidate gene and QTL mapping), and were recorded in their respective categories as such (candidate gene, QTL mapping, or GWAS). For analyses documenting overall descriptive trends (e.g., species assayed) in the literature, these studies were included only once.

2.3.2 Data extraction

Regardless of the analytical approach used, we extracted the following data from each study: year of publication, journal of publication, taxonomic group and species, source of population (i.e. wild, semi-wild, or captive), number of populations, sample size, sex (female, male, or both), age or developmental stage of individuals tested (infant, juvenile, adult, or both juvenile and adult), behavioural trait(s) measured, and whether a significant effect was found (yes or no) as determined by study authors and equivalent to a significance threshold of $P = 0.05$. We assigned species to one of five major taxonomic groups: mammals, avian, fishes, herpetofauna (i.e. amphibians and reptiles), and invertebrates. Sample size was determined from the total number of individuals for which both genetic and behavioural data were obtained.

We identified a total of 192 behavioural trait descriptors, and as many were highly detailed (e.g., engagement with object and female response to male signal), we classified behavioural traits into functional categories while still maintaining the fine-scale distinction of each trait measured. We used sets of behavioural categories as provided in meta-analyses of the repeatability and heritability of behaviours (Bell et al. 2009 and Dochtermann et al. 2019, respectively), with behaviours assayed assigned to one of 14 functional groups (Supplemental Table A.2).

For studies using a candidate gene approach, we further extracted the specific gene(s) within which allelic variation was investigated. As population stratification is known to influence association tests by generating spurious false positives (Laird and Lange 2011), we additionally recorded whether or not (yes or no) candidate gene study authors reported accounting for population structuring or relatedness between individuals.

For QTL mapping studies, we recorded the type of population used: inbred line cross (i.e. F2-cross or backcross designs) or pedigreed population. In addition, we recorded the marker type used (amplified fragment length polymorphism (AFLP), random amplified polymorphism DNA (RAPD), microsatellites, and/or SNPs), as well as the number of markers used in analyses. Often, QTL mapping identifies larger chromosomal regions composed of many genes (Allendorf et al. 2012); therefore, we determined the frequency of studies that used fine-scale mapping and/or annotated regions in an attempt to narrow down the search for candidate loci. For both QTL mapping and GWAS approaches, we recorded whether the behavioural trait measured appeared to be polygenic or oligogenic as determined by the author(s) of each study. Similar to QTL studies, we determined the number of genomic markers used in GWASs, and whether study authors attempted to annotate significant loci discovered.

2.3.3 Data analysis

We summarized the following information for all approaches combined and/or for all analytical techniques individually: a) taxonomic spread and species focus of studies; b) sample size; c) developmental stage and sex of individuals tested; d) behavioural categories assayed; e) candidate genes tested; f) candidate gene studies that corrected for population stratification; g) number and type of genetic markers used; h) determination of polygenic/oligogenic nature; and, i) fine-scale mapping or genome region annotation.

We further recorded test statistic values as reported by study authors. For candidate gene studies, we preferentially selected reported F, t, and χ^2 statistics over *P*-values, but used *P*-values if the aforementioned statistics were not provided. Four candidate gene studies in our dataset used a comparative species approach and pooled up to 23 species in a single analysis; as such, we excluded these comparative studies in our meta-analyses. Logarithm of odds (LOD) scores were extracted from QTL mapping studies, and then converted to *P*-values according to Nyholt (2000). The *P*-value of the most significant SNP for genome-wide association analyses was recorded as reported by study authors, and if not provided in the study, the value was estimated from Manhattan plots. Following Coltman and Slate (2003), each statistic was converted into a correlation coefficient (r), and subsequently transformed using Fisher's *z*-transformation (*Zr*) for all analyses.

To assess the influence of taxonomic differences on effect size, as well as the effect of various moderating variables (e.g., behaviour and source of population tested), we performed phylogenetically-controlled mixed-effect analyses using the metafor package in R ver. 3.5.3 (R Core Team 2019; Viechtbauer 2010). Adopting the approach outlined in Dochtermann et al. (2019), we generated a phylogenetic meta-regression model, initially including only an intercept and random effects (article ID and species). A phylogenetic tree of species in our database was constructed using the R package rotl (version 3.0.9) (Michonneau et al. 2016), which harvests data from Open Tree of Life (https://tree.opentreeoflife.org/curator) (Hinchliff et al. 2015). From this global model, and using the phylogenetic tree, we calculated total dataset heterogeneity and estimated sources of dataset variation due to article identity, phylogeny, and sampling according to Nakagawa and Santos (2012) (see Dochtermann et al. 2019). Similar to Dochtermann et al. (2019), a caveat to our analysis was that *Zr* was not normally distributed; yet, the *Zr* transformation was most appropriate for our meta-analysis objective.

We next fit the model with moderators as fixed effects, while keeping the same random effects as before and also accounting for phylogenetic information. The fixed effects included taxa (avian, fish, invertebrate, or mammal), source of population (captive, semi-wild, or wild), behavioural category (Table A.2), and analytical approach (candidate gene, QTL mapping, and GWAS). To determine the significance of moderators, we performed likelihood ratio tests comparing models with and without the term of interest.

We assessed the issue of publication bias towards studies with large and significant effects by constructing a funnel plot to visualize the distribution of effect sizes and precision estimates, as well as used an Egger's regression test (Egger et al. 1997). Lastly, we employed a trim-and-fill technique to determine whether studies were potentially missing from our dataset. All statistical analyses were conducted in R ver. 3.5.3 (R Core Team 2019).

2.4 Results

Our search criteria yielded 149 total studies (Table A.3), wherein we identified 841 candidate gene estimates from 85 studies, 415 QTL mapping estimates from 50 studies, and 24 GWAS estimates from 17 studies. We refer to an "estimate" as an instance where a study assessed and reported on various behavioural traits, populations, sex, species, and/or markers or loci identified. Three papers examined more than one analytical technique (Johnson et al. 2015,

Liu et al. 2015, and Santure et al. 2015). Published articles were scattered over 59 scientific sources. *Molecular Ecology* and *Behaviour Genetics* were the most well-represented journals; yet, the number of articles found in these two journals accounted for only 20.1% of total studies in our dataset, demonstrating the large spread of papers among sources (Table A.4). The number of behavioural studies has generally increased over time for each technique used, with an apparent jump in the number of studies in 2012 (Figure 2.1). However, a noticeable decline in the number of studies has occurred since 2015. In general, articles on candidate genes dominated the literature across years, followed by QTL mapping approaches. Not until 2013 is it apparent that the GWAS approach became a viable or attractive option for studying the genetic basis of behavioural variation in natural systems. Data collection for the year 2018 ceased in early July; therefore, results are only reported for studies published in July 2018 or earlier.

2.4.1 Taxonomic spread

Publications focused on species representing mammal, avian, fish, and invertebrate taxa (26.8%, 28.2%, 28.2%, and 16.8% of studies, respectively). Surprisingly, no papers were found that assessed the genetic basis of behaviour in herpetofauna. Nearly all of the candidate gene studies dealt with mammal (43.5%) and avian (43.5%) species, with few studies representing fishes (7.1%) and invertebrates (5.9%). Alternatively, fishes and invertebrates were the focus of many more QTL mapping studies (50% and 38%, respectively), compared to mammal (8%) and avian (4%) taxa. Studies on fish dominated the GWAS literature, comprising 70.6% of studies (avian, 23.5%; invertebrate, 5.9%) (Figure 2.2).

Articles were published on 13 unique species of mammals, 55 species of birds, 21 species of fishes, and 12 species of invertebrates (Table A.3). The most commonly studied group of mammals included non-human primates (67.4% of species represented in studies), while rodents represented 30.2% of mammals. We found only one study that focused on a species of mammal that did not fall under one of these broad family classifications [bighorn sheep, *Ovis canadensis* (Poissant et al. 2013)]. Most species of birds were passerines, with great tits (*Parus major*) the most widely studied individual avian species (18.3%) and species/subspecies of the genus *Junco* made up 14.6% of bird species. Of all fish species, studies were predominantly on rainbow and steelhead trout (*Oncorhynchus mykiss*; 37.5%), and over a third of all invertebrate studies focused on the honey bee (*Apis mellifera*; 34.8%) (Table A.3).

2.4.2 Sample size, developmental stage, and behaviours

The sample size used for conducting candidate gene association tests ranged from 4 to 2398 individuals (median = 101 individuals). Sample sizes for QTL linkage analyses varied from 26 to 2148 individuals (median = 198 individuals), and that for GWASs ranged from 78 to 2045 individuals (median = 228 individuals) (Figure A.1).

Over half of studies performed research solely on adults (55%), while 16.1% of studies focused on both adults and juveniles. Of all studies, 8.7% were on juveniles alone, 4% on young or infant individuals, and 16.1% of studies did not report the developmental stage or age class of sampled individuals. In addition, most studies considered both sexes (71.6%), while 12.5% and 5.1% of studies considered only males or females, respectively. 10.8% of studies did not report the sex of individuals tested.

Studies assessing the genetic basis of behaviour most frequently reported estimates for commonly studied personality traits (32.3%; activity, 5.3%; aggression, 2.4%; boldness, 7.8%; exploratory, 12.2%; social behaviour, 4.6%), followed by reproductive behaviour (14.9%) and migratory/dispersal behaviour (8.7%). Various behavioural traits classified as "other" also accounted for a large proportion of estimates (17.9%). Per individual analytical approach, personality traits were found to be highest in candidate gene and QTL mapping studies (35.9% and 25.9%, respectively), whereas migratory/dispersal behaviour dominated the GWAS literature (70.8%).

2.4.3 Candidate gene

Of the 85 candidate gene papers, 51.6% examined free-ranging populations, 33% investigated populations of captive animals, and 15.4% of semi-wild individuals. A total of 49 candidate genes and/or multigene families (taste receptor and major histocompatibility gene complexes) were examined in studies pulled for analysis. Dopamine receptor D4 (*DRD4*), serotonin transporter (*SERT*), arginine vasopressin receptor 1a (*Avpr1a*), circadian locomotor output cycles kaput (*CLOCK*), and adenylate cyclase activating polypeptide 1 (*ADCYAP1*) represented the top five genes tested (Table 2.1) (refer to Table 2.1 for top ten genes and Table A.5 for the full list of genes with corresponding behaviours tested). The vast majority of studies focused attention on testing the effects of one candidate gene (70.6%; 60 studies), while 15.3% (13) and 14.1% (12) of studies tested two or three or more genes, respectively. Most candidate

gene studies (63.5%) did not report correcting for population structure or relatedness when testing for a phenotype-genotype relationship. Conversely, 32.9% of studies did account for stratification, while 2.4% of studies mentioned expectations of it, and in 1.2% of studies, correcting for stratification was not applicable as a monomorphic marker was identified.

2.4.4 QTL mapping

Most QTL mapping studies performed a backcross or F2-cross (45 of 50 studies). We found five articles that used detailed pedigree information, only two of which from individuals of wild populations: bighorn sheep (*O. canadensis;* Poissant et al. 2013) and the great tit (*P. major*; Santure et al. 2015). The number of genetic markers used in QTL mapping studies ranged from 54 to 186,984 markers (median = 330 markers) (Figure A.2). The marker of choice for QTL mapping analyses was predominantly microsatellites (22 studies) and SNPs (20); yet, RAPDs (3), AFLPs (14), and an x-linked marker (1) were also used, either alone or in combination with other markers. Half of all studies reported finding a polygenic basis underlying the trait assayed, as opposed to 12% that reported an oligogenic effect. One study reported both a polygenic and an oligogenic effect. The remaining studies did not indicate whether the trait was polygenic or oligogenic (32%), but did determine a pleiotropic effect (4%). Lastly, most QTL studies did not perform fine-scale mapping or attempted annotation of suggestive/significant genomic regions detected (64% of studies).

2.4.5 GWAS

Of the 17 GWAS studies, 14 were on individuals from the wild, two were of wild-origin (field colonies and artificially spawned), and one of captive individuals. The number of markers used for GWASs ranged from 2593 to 459,502 SNPs (median = 10,415 SNPs) (Figure A.2). Similar to QTL mapping results, 41.18% suggested a polygenic basis of traits and 11.76% an oligogenic basis. One study reported both, and 41.18% of studies did not conclude either. All but two studies attempted annotation or mapping techniques to identify candidate loci.

2.4.6 Association effect and effect of moderators

We found that most authors of candidate gene studies did not report a significant association between the trait of interest and genetic marker(s) tested (74.7% of estimates). On the

other hand, QTL mapping results revealed that 49.6% of estimates did find a significant effect, while 30.4% and 20% found a suggestive genomic region or no effect, respectively. Of those that performed a GWAS, most (79.2% of studies) found a significant relationship between at least one marker and a behavioural trait.

Heterogeneity in the dataset was high ($I^2 = 98.94$), with sources of variation due to article (91.05%), phylogeny (7.89%) (Figure A.3), and sampling error (1.06%). The source of population significantly influenced the magnitude of resulting effects ($\chi^2 = 11.09$, df = 21, $P = 0.011$). Captive, controlled cross, and semi-wild populations had the highest average effect size estimates ($Zr = 0.26$ [95% confidence interval (CI): 0.20:0.31], $Zr = 0.3$ [0.24:0.36], and $Zr = 0.34$ [0.20:0.46], respectively), whereas populations of wild origin had a moderate effect ($Zr = 0.16$ [95% CI: 0.11:0.21]). In addition, significant variation was found among the molecular approach undertaken ($\chi^2 = 16.44$, df = 22, P = 0.0003) (Table A.6). The average effect size for candidate gene studies ($Zr = 0.16$ [95% CI: 0.11:0.21]) was lower than QTL mapping ($Zr = 0.3$ [95% CI: 0.25:0.35]) and GWAS ($Zr = 0.39$ [95% CI: 0.33:0.46]) approaches (Figure 2.3) (Table A.7).

Taxonomic group to which the assayed organism belonged to did not contribute significant variation in test effects ($\chi^2 = 4.17$, df = 21, $P = 0.24$), nor did behavioural category screened ($\chi^2 = 10.6$, df = 11, $P = 0.64$) (Table A.6). For all but one behavioural category (sleep behaviour), the average effect sizes ranged from 0.21 to 0.26 (Figure 2.3) (Table A.7). The average effect size estimate of sleep behaviour had particularly wide error bars (Figure 2.3), consistent with this behavioural category being weakly represented in the dataset with estimates drawn from two studies.

2.4.7 Publication bias

Visual inspection of the funnel plot indicated some evidence of publication bias with respect to studies of smaller precision having a larger effect (Figure 2.4). Results from Egger's regression tests substantiates the presence of this bias ($t_{1061} = -18.76$, $P < 0.001$) in the literature. Lastly, the trim-and-fill method indicated that the number of missing biased estimates in our dataset was zero (SE = 16.89).

2.5 Discussion

Understanding the genetic basis of animal behaviour in natural systems is undoubtedly complex, but increased accessibility of molecular genetic approaches now facilitates unravelling the processes shaping and maintaining interindividual behavioural variation in non-model organisms. Building upon other reviews and meta-analyses assessing research efforts in identifying loci underpinning behavioural variation, our analysis shows that the recognition of the feasibility of performing molecular genetic research of behaviour in natural systems (e.g., Bell 2008; Boake et al. 2002; Laine and van Oers 2017; van Oers and Mueller 2010) is being met with an increased number of studies. However, given the large number of studies that exist describing behavioural variation and animal personality, assessing the molecular genetics underlying behaviour remains in its infancy. In addition, our results revealed interesting and notable trends in the literature including limited taxonomic breadth, few behavioural categories examined, and trends in the preferred molecular approach(es) undertaken.

2.5.1 Literature trends

In 2011, Carere and Locurto reported that individual behavioural differences have been described for more than 100 species, a number now likely underrepresented given the explosion of published studies on interindividual behavioural variation over the last 15 years (Bengston et al. 2018; Roche et al. 2016). Despite this, our findings indicate a relatively narrow and biased focus on the type and number of species for which such behavioural data is examined genetically. Regardless of molecular approach undertaken, we found that certain taxonomic groups are over-represented in the literature, while organisms belonging to taxa like the herpetofauna are not represented at all. Of all major taxonomic groups, studies on birds were especially prevalent with the highest number of unique species observed (52 of 84 species), although most are from a single order, the Passeriformes. Only 21 fishes, 13 species of mammals, and 12 species of invertebrates were the target of research. Such skew and low numbers of unique species could be a reflection of the relative ease of conducting behavioural research of certain taxa, such as birds, in the wild (Fidler 2011). The taxonomic bias observed here has also been documented in the behavioural literature (Dochtermann et al. 2019; Rosenthal et al. 2017), as well as in other areas of research [e.g., reintroduction biology (Seddon et al. 2005) and conservation research (Creighton and Bennett 2019)]. A narrow species focus presents

problematic consequences for the study of animal behaviour, notably a misunderstanding of the universality of ecological and evolutionary processes underlying behaviour. Moreover, applying invalid broad-scale behavioural processes inferred from a limited set of taxonomic diversity to all taxa has direct implications for wildlife conservation and management, as well as incorrectly drawing inferences and developing evolutionary theories (Rosenthal et al. 2017). As such, to fully understand the processes creating, maintaining, and shaping behavioural variation of animals in natural populations, we stress the need for more taxonomic breadth in elucidating the genetic basis of behaviour.

Animal personality research has gained widespread attention in recent decades (Carere and Maestripieri 2013); yet, despite the relative infancy of the field, we observed that the largest proportion of behavioural studies in our dataset focused on commonly studied personality traits (activity, aggression, boldness, exploratory, and social behaviour) (Réale et al. 2007). This finding corroborates an ever-growing acknowledgement and acceptance of animal personality (Roche et al. 2016), and moreover, it seems likely that the high proportion of personality studies observed here reflects a movement of animal behaviourists following suit with human personality-genetics research. Coincident with the popularity and polarizing topic of animal personality (Roche et al. 2016), developments in human and laboratory organism research set the stage for taking a genetic perspective of personality in natural populations, for example as candidate genes were identified and methodologies refined (Savitz and Ramesar 2004; Terracciano et al. 2008). Yet, the field of animal personality has been met with opposing viewpoints about terminology usage, validity of traits measured, and inappropriate behavioural assays (Réale et al. 2007; Carter et al. 2013) that has led to a blurred and often debated distinction between personality and other behavioural traits (e.g., Beekman and Jordan 2017). A focus on personality-genetic research does not necessarily preclude progress made in our understanding of other behavioural categories. Personality traits are tightly integrated and influence the outcome of other behavioural patterns (Réale et al. 2007), including foraging, migration and dispersal, reproductive strategies, and parental care. Nevertheless, research of the other behavioural categories is fundamental to substantiate behavioural functional links, and to further identify underlying common biological pathways to aid in the inference of evolutionary patterns. Interestingly, we found that, while collectively studies on personality traits were found in greater proportion than other behavioural categories, the most widely researched behavioural

trait varied according to molecular method used, such that the primary focus of GWASs to date has been on migratory behaviour while QTL mapping and candidate gene studies were largely on personality.

It is not surprising that studies using a candidate gene approach dominated the literature given its accessible use in natural populations without the need for a detailed pedigree. However, we predicted that GWAS would have replaced QTL mapping methods in later years, a notion only realized since 2015 (Figure 2.1). QTL mapping is a restrictive method, in that it requires controlled crosses or a pedigree, and as such does not always lend itself for use in unmanipulated or wild animals. In addition, effect sizes of QTL mapping studies are often overestimated, an issue due to the Beavis effect (Beavis 1994; Slate 2013). Given these restrictions, we anticipated that recent progress in sequencing, bioinformatic, and statistical techniques would facilitate greater use of GWAS in natural populations. Unlike QTL mapping, GWAS does not require a pedigree and offers better resolution of QTL positions, identifying the genomic regions or, in rare cases, the causal genes underlying phenotypic variation. Nevertheless, GWAS is not without its own limitations. Part of what makes GWAS attractive- higher resolution- requires a high-density panel of genetic markers so that a marker will be in strong linkage disequilibrium with a causative variant and thus allow detection of an association (Slate et al. 2010). Such marker sets are likely not available for many species, though new methods for generating panels of SNPs in nearly any species (e.g. restriction-site associated DNA sequencing; Baird et al. 2008; Miller et al. 2007) may change this going forward. In addition, to detect true associations, sample size needs to be sufficiently large enough to provide power, a requirement not always feasible for studies of free-ranging populations.

A variety of research is now confirming that many quantitative traits have an underlying polygenic architecture (Santure and Garant 2018), a theory accepted by researchers to also extend to behavioural traits, although this has not been explicitly tested (Laine and van Oers 2017). In support of this, we found that the majority of authors of behavioural mapping studies (both GWAS and QTL) concluded a polygenic trait architecture. The polygenic nature of complex traits can create challenges for association studies, however. Association approaches are biased towards the detection of large effect loci, and furthermore, the effect sizes of loci that do reach statistical significance can be inflated. In addition, because many loci are expected to affect phenotypic expression, genotype frequencies that vary across time, geographical scales, and

distinct environments may result in false positive associations (Santure and Garant 2018; van Oers and Mueller 2010). However, analytical methods have recently been developed to address these challenges in an effort to reduce false positive error rates (see Santure and Garant 2018). Given the costs/benefits of both GWAS and QTL mapping, it will be interesting to see if the trends we find continue into the future.

2.5.2 Meta-analysis of literature

From our meta-analysis, we found no evidence that effect size varied among the taxa represented (avian, mammal, fishes, and invertebrates), nor did we find a strong phylogenetic signal, suggesting that relatedness did not account for a significant amount of heterogeneity among studies (Figure A.3). Nevertheless, because of the over-representation of certain taxa in our dataset, it may be difficult to detect a phylogenetic signal with biased taxonomic coverage (Dochtermann et al. 2019). This corroborates our earlier call for the need for research on taxonomically diverse organisms, including the less-studied species that make up a large percentage of all biodiversity.

Additionally, we found no support that effect size variation was driven by the behavioural category tested. Even so, communication, social, and migratory/dispersal behaviours exhibited the highest effects observed, with boldness and foraging also among categories with higher average effect sizes (Figure 2.3). In their meta-analysis of the heritability of behaviour, Dochtermann et al. (2019) found that behavioural category examined by researchers significantly influenced heritability estimates. The authors further reported that among other behavioural categories, migratory/dispersal and communicative behaviours had high narrow sense heritability estimates (h^2 = 0.456 and 0.351, respectively). While we did not find that average effect size varied significantly with behavioural category, our results do support those of Dochtermann et al. (2019) with respect to the genetic influence on these particular behavioural categories (Figure 2.4).

Conversely, we found that the population origin of individuals tested did influence variation in effect size estimates, a finding that may be explained by the different environmental variables experienced by captive, semi-wild, or wild organisms. That the development of behaviour has an important environmental component is not new (Bateson 1979), but to what extent behaviour is shaped by genes, environment, and the interaction between the two remains

to be seen. We found that tests in captive and semi-wild populations had a higher genetic effect than those of wild populations across behavioural categories examined. Individuals of captive populations are often exposed to less environmental flux, and such stability in captivity has been shown to relax selection pressures over time (Stamps and Groothuis 2010). This could potentially lead to reduced phenotypic variation in captive populations. In addition, genetic variability in captive populations may be reduced due to the founder effect. Together these factors may inflate effect sizes when an association is observed. It is also possible that a lesser effect in wild populations is the result of selection for "an optimal trait", leading to less trait variance and thus making detection of an association more difficult. Still yet, the difficulty associated with measuring animal behaviour in the wild, when compared to controlled and more easily accessible captive settings, may lead to less precise measurements of behavioural phenotypes, yielding lower effect sizes. These results add to long-standing questions (e.g., Bell and Aubin-Horth 2010): are certain behaviours more sensitive to environmental flux; and, is there any inference as to which behaviours appear to be influenced more by genetic or environmental factors? The relationships between environment, population source, and behaviour deserve further investigation.

Lastly, we found that molecular approach strongly affected effect size variation. Specifically, QTL mapping and GWAS studies reported particularly high effects relative to candidate gene studies. This was not unexpected as QTL mapping studies are known to be widely influenced by the Beavis effect (Slate 2013), often leading to inflated QTL effect sizes. We further acknowledge that by pulling statistics for the most significant locus from GWAS analyses, we likely biased the effect sizes upwards by not including the null associations for this approach. Moreover, and despite the cross-species utility of candidate genes known to influence behavioural variation (Fitzpatrick et al. 2005), candidate gene association tests have been met with mixed results between and among species and populations (e.g., Korsten et al. 2010). To add, the median estimate of sample size for candidate gene studies was approximately half that of mapping studies, and therefore could contribute to lower effect sizes by influencing study power; although, small studies tend to report higher effect sizes due to reporting bias. Regardless of approach, small effect sizes are nonetheless useful in identifying biological pathways underlying traits of interest (Laine and van Oers 2017).

2.5.3 Challenges

Linking genes with phenotypic variation in free-ranging systems has been met with more limited success, in addition to heterogenous results within and between populations, than anticipated. However, this pattern mirrors that seen in model and domestic organisms (including humans) where attempts to link phenotype with genotype are often met with mixed success, including "missing heritability" at the genetic level (e.g., Yang et al. 2010; Manolio et al. 2009). Despite this, recent findings on the study of the molecular genetic basis of other complex phenotypes are encouraging. For example, linkage and association mapping studies of natural animal populations have had some success with identifying genetic variants linked with adaptation in traits such as coat coloration (Steiner et al. 2007) and climate adaptation (Garcia-Elfring et al. 2019). Lessons can be learned from these studies, as well as from an increasing number of behavioural studies that are striving to address commonly encountered issues by replicating across populations (Korsten et al. 2010), using complementary molecular approaches (Bendesky et al. 2017; Santure et al. 2015), accounting for phenotypic plasticity by modelling environment*SNP interaction terms (Gienapp et al. 2017b), and testing the validity of behavioural phenotypes (Beckman and Biro 2013). Lesson can also be taken from agronomic production where novel methods like genomic selection are being used to dissect the genetic architecture underlying complex, polygenic traits such as growth (Meuwissen et al. 2001; Robertsen et al. 2019). However, with genomic selection methods, the goal is to determine the genetic merit of individuals rather than specific QTL loci. These data could then be used in conservation efforts, such as to select individuals for translocations or reintroductions on the basis of genetic merit.

That being said, challenges remain for the study of the genetic basis of behavioural diversity. Among them, thus far few studies have attempted to replicate genotype-phenotype associations in natural populations (reviewed in Schielzeth et al. 2018), a finding likely fueled by publication bias. When attempted, however, a lack of reproducibility between analyses is often attributed to methodological and laboratory differences (e.g., sampling variance, marker density, and sampling design), but is also hampered by the biological properties of specific populations, available resources, and logistical constraints (Schielzeth et al. 2018). Identifying the genes underlying behavioural variation is further complicated with inherent issues in obtaining reliable phenotypic data. For instance, incongruous behavioural testing, scoring, and interpretation made

by researchers can influence measurements (Carter et al. 2013), and thus mask the effect of genetic variants on an intended trait. Behavioural traits are also plastic, such that environmental and developmental variables can produce shifts in behaviour across contexts, further complicating the collection of reliable data. Any environmental, spatial, and temporal effects that are left unaccounted for may contribute to the difficulty in detecting genotype-behaviour associations. Moreover, wild animals are difficult to behaviourally phenotype in numbers large enough to provide statistical power to the study (Snyder-Mackler and Tung 2017), a problem exacerbated in smaller populations.

2.5.4 Continuing role for genomics, longitudinal studies, and best practices

Until recently, using association mapping techniques to identify genetic regions underlying phenotypic variation was not possible for many organisms given limited genomic resources (Slate et al. 2010). With progress made in sequencing technologies, generating genetic data is no longer a limiting factor for such studies, notwithstanding budgets and personnel with genetics and bioinformatics expertise (Taylor et al. 2017). However, advancements in sequencing methods, such as the family of restriction-site associated DNA (RAD) sequencing methods (Andrews et al. 2016; Campbell et al. 2018), permits simultaneous SNP discovery and genotyping in the population of interest of any species at relatively low-cost. While roadmaps for navigating RAD sequencing analytical pipelines exist to provide ease to researchers during the data analysis process (e.g., Rochette and Catchen 2017), bioinformatics is nontrivial and access to expertise may be a limiting factor for research groups (Taylor et al. 2017). Alternative targeted genomic approaches, such as amplicon sequencing and sequence capture, can be performed at even lower costs, requiring less expensive and specialized laboratory equipment, and data analysis can be done with more user-friendly, standardized analytical pipelines in comparison to methods like RAD sequencing (Meek and Larson 2019). These approaches may further be enriched for potentially functional genetic variants, and are thus promising for researchers wishing to explore the gene-behaviour relationship in natural populations.

Still other avenues of research for investigating the genetic basis of behavioural diversity are emerging. In addition to allelic variation, direct effects of environmental differences between individuals could alter gene expression (Bell and Aubin-Horth 2010). That is, the environment experienced by an individual has the capacity to shift trait expression despite the individual's

genetic makeup. Therefore, advances in transcriptome sequencing and microarray development (e.g., through use of expressed sequence tags) enables assessing whole-genome expression with the aim of identifying particular biological pathways, as well as discovering new candidate genes involved in the generation and maintenance of behavioural variation and plasticity (Bell and Aubin-Horth 2010). Further, some studies are finding that epigenetic mechanisms, such as DNA methylation, trigger changes in gene expression that subsequently affect behavioural variation (e.g., Verhulst et al. 2016). These genomic structural modifications can be the consequence of rapid changes in the environment and are potentially transmitted to subsequent generations. As the environment may have a greater impact on behaviours in the wild, whole-genome expression and epigenetics are exciting areas of behavioural research.

Long-term research of individually recognizable or marked animals have made possible studies of the quantitative genetics of behavioural traits in wild populations (Clutton-Brock and Sheldon 2010a). Such longitudinal individual-based records have the potential to play an invaluable role in the dissection of the genetic architecture of complex quantitative traits. Studies exist that span multiple generations and include records for hundreds to thousands of individuals (Slate et al. 2010), and may have archived samples/banks of stored tissues. Few studies analyzed for this review are from well-known longitudinal studies [the great tit (e.g., Santure et al. 2015) and bighorn sheep (Poissant et al. 2013)], but the possibility for other systems from currently under-represented taxa with which long-term behavioural datasets and banks of stored tissues exists. We are aware of studies with exceptional longitudinal datasets on species such as African lions (*Panthera leo*) (Packer et al. 2005), grey seals (*Halichoerus grypus*) (Bowen et al. 2006; Bubac et al. 2018), red squirrels (*Sciurus vulgaris*) (Boon et al. 2008), spotted hyenas (*Crocuta crocuta*) (Van Horn et al. 2004), and monarch butterflies (*Danaus plexippus*) (McCord and Davis 2010), to name a few, that would greatly advance our understanding of the genetic basis of behavioural traits if genotype could be linked with phenotype. Combining longitudinal datasets and archived samples with the genomic methods discussed above has the potential to unlock the next wave of behaviour-genomics.

Lastly, we urge researchers to report all necessary components of analysis and/or information that would aid future meta-analyses. Specifically, authors should clearly state sample sizes used in analyses and provide statistic values and/or effect sizes for all test results, including loci of no effect. For reviews and meta-analyses wishing to identify patterns and

underlying pathways, it would be helpful if authors clarified biological variables of test subjects including developmental stages, sex of individuals, and temporal/geographic scales. For candidate gene studies, as well as for GWASs, it is important to correct for population stratification/bias to avoid false positive associations and inflation of results, leading to incorrect broad-scale assumptions. Finally, as echoed across many disciplines, we encourage authors to pursue publication of null results, a notion easier said than done. We found evidence for publication bias in the literature, indicating how integral it is for studies of no effect to reach journals of impact for our complete and undistorted understanding of the genetic influence on behavioural variation.

In conclusion, despite the challenges faced thus far, we think there is value in continued efforts aimed at shedding light on evolutionary processes shaping and maintaining behavioural variation in wild systems. As we continue to unravel the genetic basis of behaviour in natural populations, we can discover the proximate mechanisms underlying behavioural variation and determine whether such mechanisms are evolutionarily conserved across species, predating lineage divergence and/or leading to similar phenotypic outcomes. Such research will continue to be essential to aid in predicting the adaptive potential of species in the face of emerging novel selection pressures in rapidly changing environments. Moreover, it will contribute to our understanding of behavioural evolution across the animal kingdom, and in doing so, will attempt to address fundamental questions in evolutionary biology. Widening the taxonomic breadth and taking advantage of longitudinal studies with existing tissue banks that can be linked with behavioural variation will be key to making these inferences.

Table 2.1: A list of the top ten candidate genes explored and the corresponding behavioural traits measured in association tests.

Gene symbol	Gene full name	Behaviours tested	# of studies
DRD4	Dopamine receptor D4	Activity; Aggression; Boldness; Communication; Exploratory; Migratory; Parental; Reproductive; Social	22
CLOCK	Clock circadian regulator	Migratory; Parental; Reproductive; Sleep	21
SERT/SLC6A4	Serotonin transporter Solute carrier family 6 member	Activity; Aggression; Anxiety/Stress; Boldness; Communication; Exploratory; Migratory; Reproductive; Social	17
AVPR1A	Arginine vasopressin receptor 1A	Activity; Aggression; Anxiety/Stress; Boldness; Reproductive; Social	14
ADCYAP1	Adenylate cyclase activating polypeptide 1	Migratory; Reproductive; Sleep	13
NPAS2	Neuronal PAS domain protein 2	Migratory; Reproductive; Sleep	8
CREB1	cAMP responsive element binding protein 1	Migratory; Reproductive; Sleep	7
TPH2	Tryptophan hydroxylase 2	Aggression; Boldness; Social	4
MAOA	Monoamine oxidase A	Aggression; Anxiety/Stress; Boldness; Social	3
AANAT	Aralkylamine N-acetyltransferase	Exploratory; Sleep	3

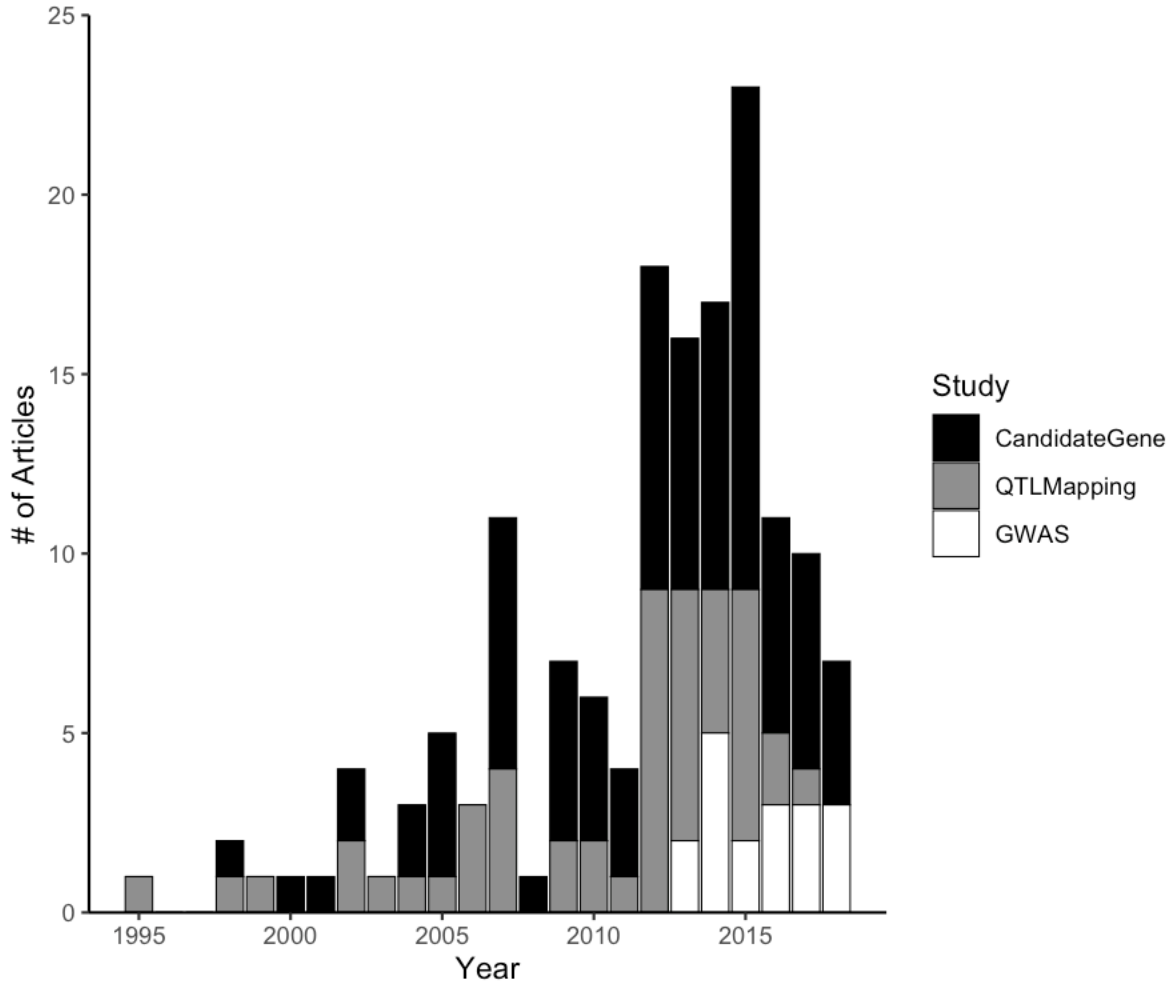

Figure 2.1: Bar graph showing the number of articles using candidate gene, QTL mapping, and GWAS approaches to study the genetic basis of behaviour in natural systems over time.

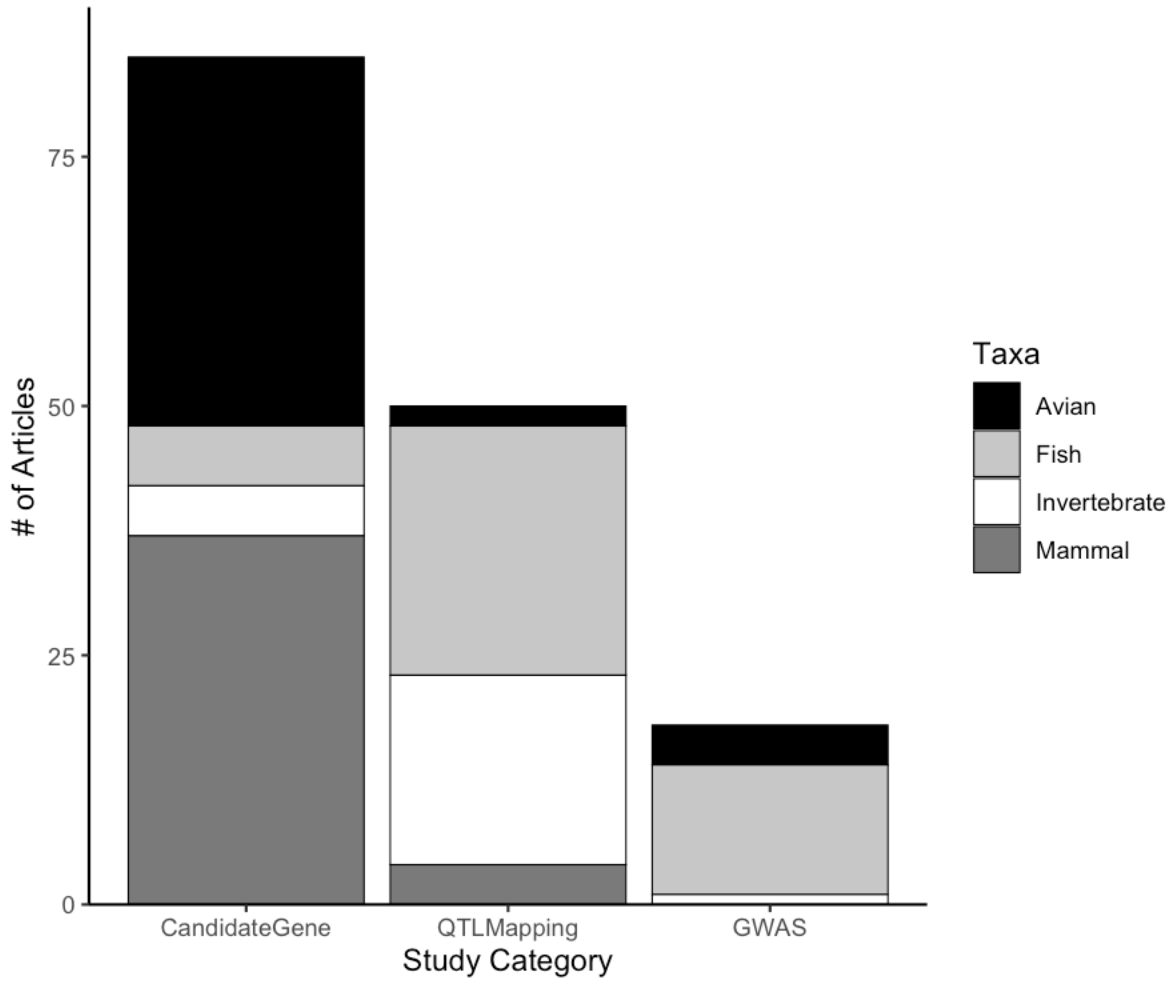

Figure 2.2: Bar graph showing a taxonomic breakdown of the number of studies published using each analytical technique to study the genetic basis of behaviour in natural systems.

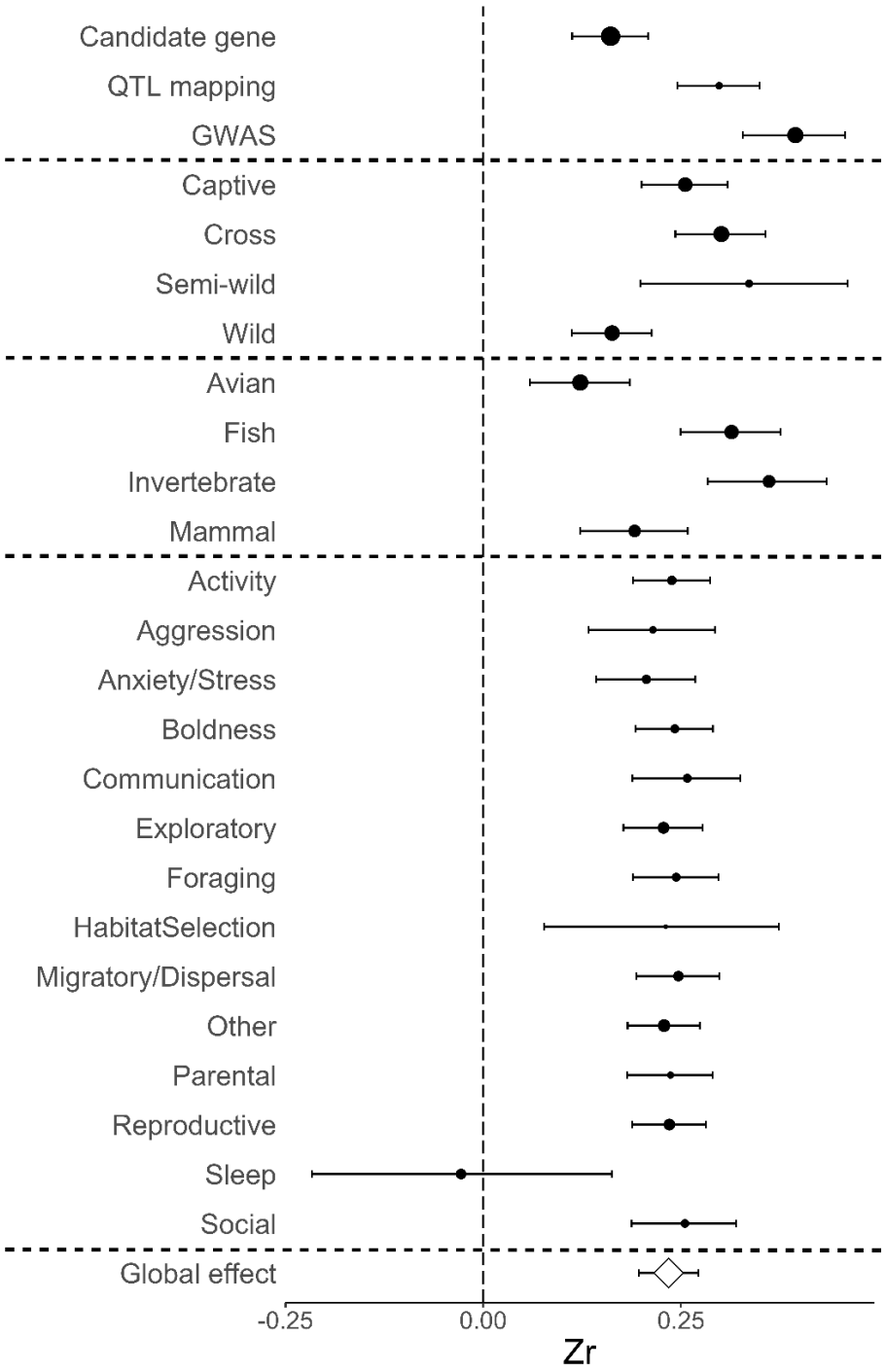

Figure 2.3: Forest plot of average effect size estimates (± 95% CIs) for each moderator tested to assess the magnitude of effect in association and linkage analyses of molecular behavioural studies. The size of the point or symbol is representative of the number of estimates included for that average effect estimate.

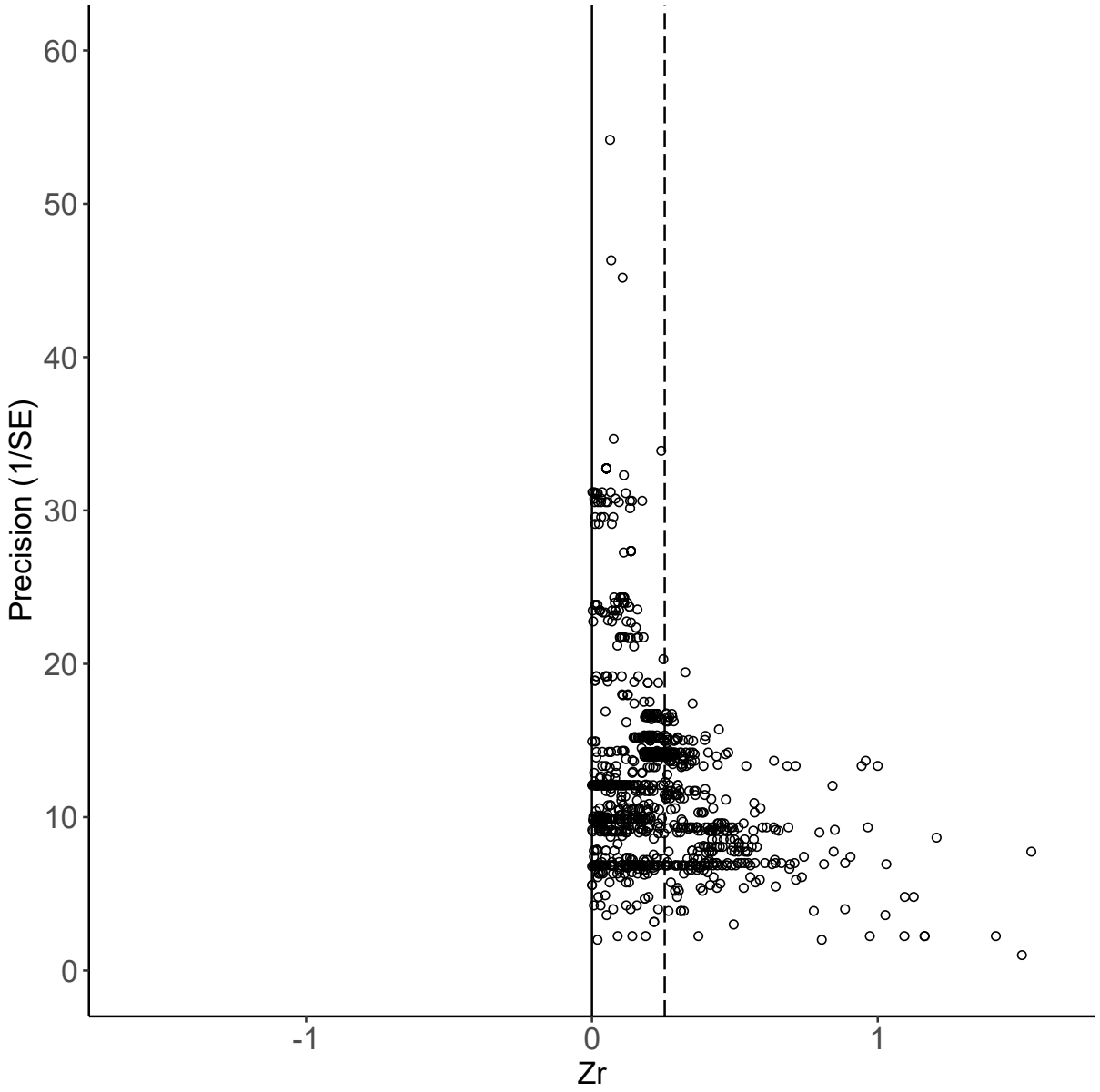

Figure 2.4: Funnel plot for the detection of publication bias in the literature assessing the genetic basis of behaviour in natural, or semi-natural, populations. The y-axis is the index of precision (1/SE) (Egger et al. 1997), and the x-axis is the effect size (*Z*r) of individual studies analyzed.

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Appendices

A.1 Additional tables

Table A.1: Results from various combinations of literature search term results using three databases: Web of Science, Scopus, and Google Scholar.

Combinations of search terms	Web of Science	Scopus	Google Scholar
“personality” AND “candidate gene”	166	296	13,500
“behav* syndrome” AND “candidate gene”	4	8	206
“coping style” AND “candidate gene”	2	5	309
“temperament” AND “candidate gene”	38	83	3660
“animal behav*” AND “candidate gene”	4	259	26,000
“personality” AND “QTL mapping”	4	3	853
“behav* syndrome” AND “QTL mapping”	0	0	34
“coping style” AND “QTL mapping”	0	0	42
“temperament” AND “QTL mapping”	1	0	337
“animal behav*” AND “QTL mapping”	2	32	9260
“personality” AND “QTL analysis”	11	7	691
“behav* syndrome” AND “QTL analysis”	2	2	41
“coping style” AND “QTL analysis”	0	1	40
“temperament” AND “QTL analysis”	3	3	247
“animal behav*” AND “QTL analysis”	6	43	7840
“personality” AND “GWAS”	69	82	7190
“behav* syndrome” AND “GWAS”	0	2	56
“coping style” AND “GWAS”	1	1	146

“temperament” AND “GWAS”	14	9	1750
“animal behav*” AND “GWAS”	3	23	8590
“personality” AND “genome-wide association”	278	213	13,400
“behav* syndrome” AND “genome-wide association”	0	2	131
“coping style” AND “genome-wide association”	2	1	296
“temperament” AND “genome-wide association”	50	46	3300
“animal behav*” AND “genome-wide association”	2	90	708

Table A.2: Categorization of behavioural trait descriptors used in summary statistics and the candidate gene meta-analysis.

Behavioural Category	Description
Activity	General activity level of an individual (e.g., movement patterns and speed)
Aggression	Agnostic behaviour exhibited towards conspecifics
Anxiety/Stress	Exhibiting apprehensive, unease, and/or nervous behaviour
Boldness	Response to a potentially risky situation or novel object/situation
Communication	Transfer of inter- or intraspecific information
Exploratory	Investigation of novel environment
Foraging	Behaviour exhibited during feeding events
Habitat selection	Selection of preferred habitat
Migratory/Dispersal	Annual movement patterns or movement from natal grounds
Parental	Behaviour exhibited during rearing of offspring
Reproductive	Mating strategies or courtship rituals
Sleep	Sleep patterns including restlessness, duration of sleep, etc.
Social behaviour	Affiliation or attraction to conspecifics
*Other	Psychological assessment traits, grooming/hygiene, dominance, learning/memory, fear/startle response, antipredator behaviour

Table A.3: List of references used in literature review according to analytical technique, with associated species, taxonomic group, and behavioural category examined (Act = Activity; Agg = Aggression; Anx/Stress = Anxiety/Stress; B = Boldness; Comm = Communication; Exp = Exploratory; For = Foraging; Hab = Habitat selection; Mig/Disp = Migratory/Dispersal; Par = Parental; Rep = Reproductive; Soc = Social).

Supplementary Information for Table A.3 and associated study references can be found at: https://onlinelibrary.wiley.com/action/downloadSupplement?doi=10.1111%2Fmec.15461&file=mec15461-sup-0001-Supinfo.pdf

Table A.4: Scatter of papers investigating the genetic architecture of natural animal behaviour published in various scientific journals between 1998 and 2018.

Total papers/journal (%)	Overall %	# of papers	Journal
4.7-10.7	37.6	56	Molecular Ecology; Behaviour Genetics; PLoS One; Ecology and Evolution; graduate theses
2.7	10.7	16	Journal of Heredity; G3: Genes, Genomes, Genetics; Current Biology; Proceedings of the Royal Society B: Biological Sciences
2	18.1	27	Animal Behaviour; Behavioural Ecology and Sociobiology; Journal of Evolutionary Biology; PNAS; Scientific Reports; and 4 others
1.4	12.1	18	Science; Behavioural Ecology; BMC Genetics; Genetics; Evolutionary Applications; and 4 others
< 1	21.5	32	Animal Genetics; BMC Evolutionary Biology; Conservation Genetics; Ecology; Nature; and 27 others

Table A.5: Full list of genes (those not included in Table 2.1) screened in the candidate gene literature with corresponding behaviours tested. With the exception of the *OXTR* gene (n = 2 studies), each gene listed here was detected in only one study (n = 1 study).

Gene	Gene name	Behaviours tested
Abpa(g)	Androgen binding protein	Aggression
Adra2a	Adrenergic receptor	Aggression
Adrbk2	Adrenergic receptor kinase	Aggression
Akr1c6	Aldo-keto reductase; hydroxysteroid dehydrogenase	Aggression
AVPR1B	Arginine vasopressin receptor 1B	Social
C3	Complement component 3	Aggression
CACNA1c	Calcium voltage-gated channel subunit alpha 1 C	Sleep
CKI(epsilon)		Sleep
CKI(epsilon)-tau		Sleep
Cyp3a11	Steroid inducible cytrochrome P450	Aggression
D2-like dopamine receptor		Other
DAT	Dopamine transporter	Activity
Dopamine/ecdysone receptor		Other
DRD1	Dopamine receptor D1	Other
DRD2	Dopamine receptor D2	Other
for	cGMP-dependent protein kinase (foraging)	Migratory/Dispersal
GREB1-L	Growth regulating estrogen receptor binding 1 L homeolog	Migratory/Dispersal
GRIA3	Glutamate ionotropic receptor AMPA type subunit 3	Sleep
H2-K	Histocompatibility region K	Aggression
Hsd3b(-4)	Hydroxysteroid dehydrogenases	Aggression
MHC class Ia	Major histocompatibility complex, class I, A	Aggression; Other
OPRM1	Opioid receptor mu 1	Activity; Aggression; Anxiety/Stress
NPSR1	Neuropeptide S receptor 1	Sleep

Octopamine receptor 1		Other
Octopamine receptor 2		Other
OmyFbxw11		Migratory/Dispersal
OXTR	Oxytocin receptor	Anxiety/Stress; Sociality; Other
Pan I	Pantophysin	Habitat selection
PCSK2	Proprotein convertase subtilisin/kexin type 2	Sleep
PERIOD2	Period circadian regulator 2 L homeolog	Sleep
Pg9	Exopolygalacturonase	Sociality
Pgi	Phosphoglucose isomerase	Migratory/Dispersal
Ptprs	Protein tyrosine phosphatase receptor type S	Aggression
SNAP25*		Anxiety/Stress; Boldness; Other
SPT-QTLs*		Reproductive
Srd5al	Steroid reductase	Aggression
t haplotype		Migratory/Dispersal
zTas2r*		Foraging

*Study specific

Table A.6: Model results testing the influence of moderators on effect size across the molecular genetic literature.

Moderator	df	AIC	AICc	LogLik	LRT	P
Taxa	21	-1510.90	-1510.02	776.45	4.17	0.24
Population source	21	-1503.99	-1503.10	772.99	11.09	0.011
Behavioural category	11	-1524.47	-1524.22	773.24	10.60	0.64
Study approach	22	-1496.64	-1495.66	770.32	16.44	0.0003
Full model	24	-1509.07	-1507.92	778.54		

Table A.7: Model-specific estimates of moderators investigated in assessing the influence on significant sources of effect variation.

	Estimate	CI lower	CI upper	
Taxa				
Avian	0.123	0.059	0.185	***
Fish	0.314	0.249	0.376	***
Invertebrate	0.361	0.284	0.434	***
Mammal	0.191	0.122	0.259	***
Population source				
Captive	0.255	0.20	0.309	***
Cross	0.301	0.243	0.357	***
Semi-wild	0.336	0.198	0.461	***
Wild	0.163	0.112	0.213	***
Behaviour				
Activity	0.238	0.189	0.287	***
Aggression	0.215	0.133	0.293	***
Anxiety/Stress	0.206	0.142	0.268	***
Boldness	0.242	0.193	0.290	***
Communication	0.258	0.189	0.325	***
Exploratory	0.228	0.177	0.277	***
Foraging	0.244	0.189	0.297	***
Habitat selection	0.231	0.077	0.374	**
Migratory/Dispersal	0.247	0.194	0.298	***
Other	0.229	0.182	0.274	***
Parental	0.237	0.182	0.290	***
Reproductive	0.235	0.188	0.281	***
Sleep	-0.028	-0.217	0.162	
Social	0.255	0.188	0.320	***
Approach				
Candidate gene	0.161	0.112	0.209	***
GWAS	0.395	0.328	0.457	***
QTL mapping	0.298	0.246	0.349	***

*P < 0.05; **P < 0.01; ***P < 0.001

A.2 Additional figures

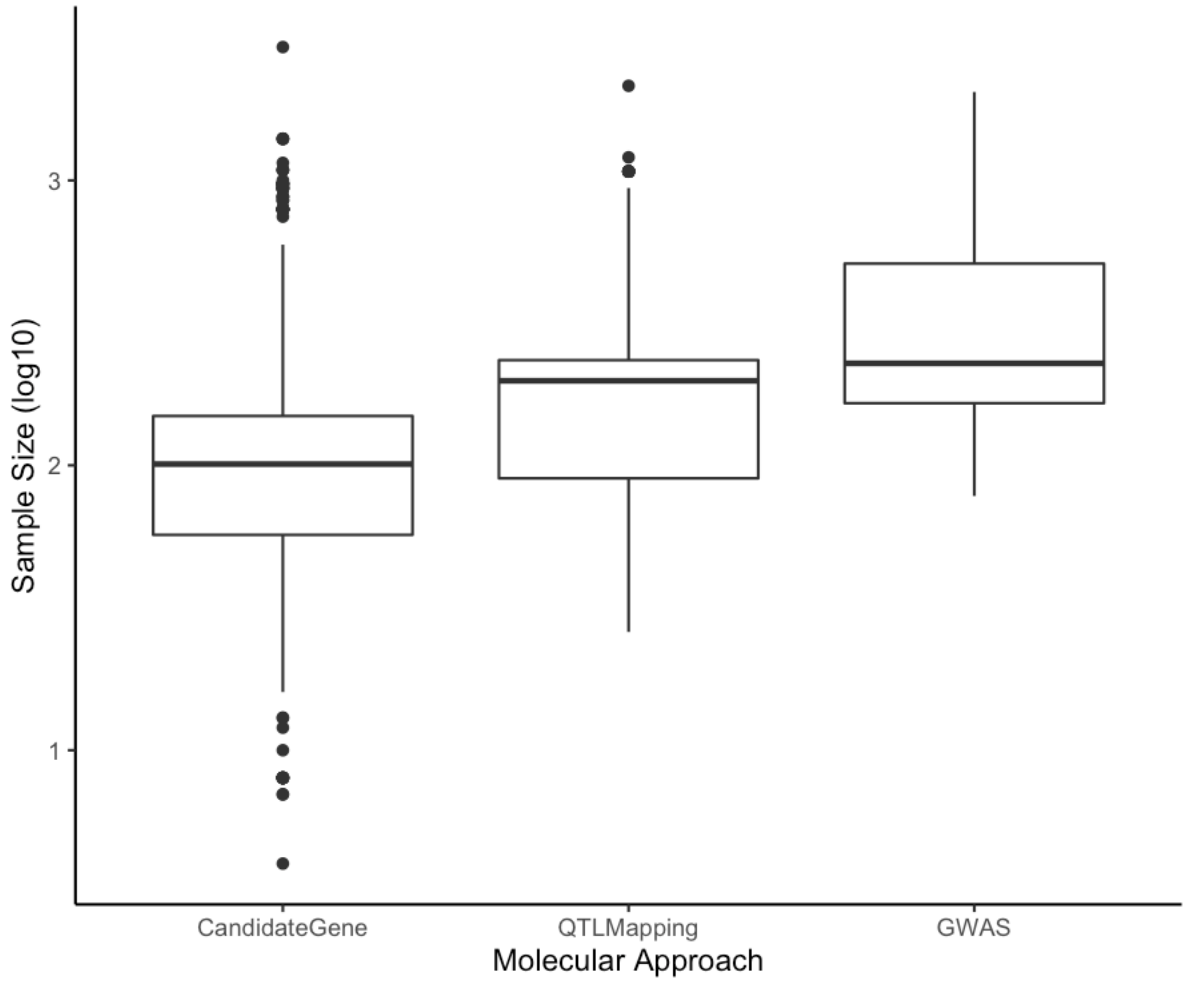

Figure A.1: Sample size of subjects for each molecular analytical method used to explore the genetic basis of behavioural phenotypes.

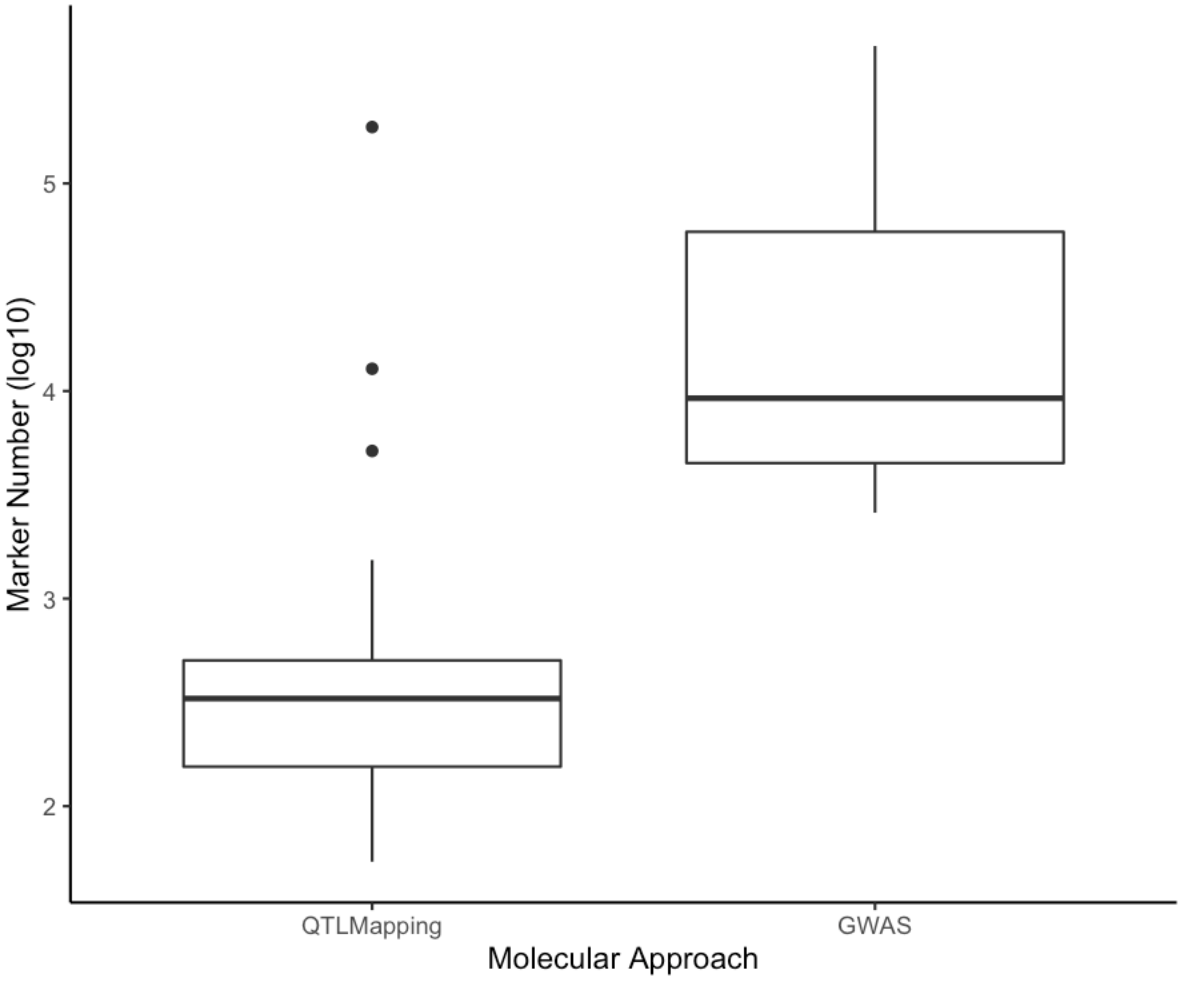

Figure A.2: Comparison of the number of markers used between quantitative trait locus (QTL) mapping and genome wide association study (GWAS) approaches.

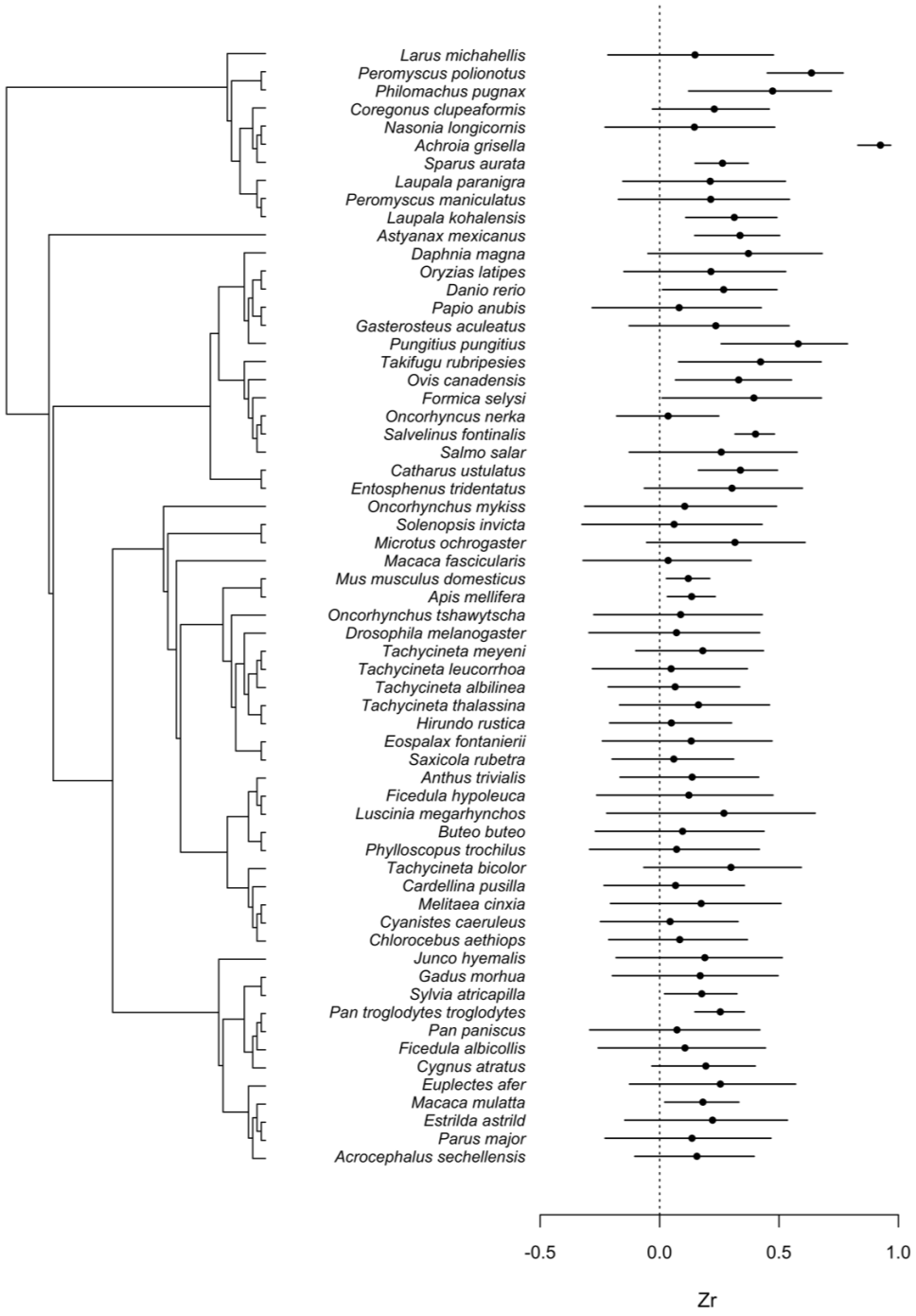

Figure A.3: Phylogenetic tree (left) and associated forest plot (right) illustrating average effect size for species analyzed in studies assessing the molecular genetic basis of behaviour in natural, or nearly natural, populations.

Chapter 3: Repeatability and reproductive consequences of boldness in female grey seals

3.1 Abstract

Wild animals show consistent individual variation in behaviour across time and/or contexts, now referred to as animal personality. While this variability may have important ecological and evolutionary implications, how and why variation in animal personality is maintained in a natural population remains unclear. In this study, we assessed the influence of environmental and biological sources of variation on behavioural responses measured along the shy-bold continuum in a long-lived, iteroparous marine mammal, the grey seal (*Halichoerus grypus*). Between 2008-2016, 469 females from the Sable Island, Nova Scotia breeding colony of grey seals were given a boldness score in response to a human approach, designed to stimulate maternal defense of offspring. Using generalized linear mixed-effects models (GLMM) in a Bayesian framework, we show that boldness is highly repeatable between and within years. There were age differences in boldness, with younger females being less bold than older, more experienced females providing some support for the life-history trade-off hypothesis. We further used GLMMs to assess sources of variation on offspring weaning mass. We found that young females that were bolder produced heavier pups than shyer counterparts, and that pups produced by bolder females were on average ~2 kg heavier than pups of shy females. These results provide further evidence that personality influences life history strategies, and illustrates the evolutionary potential of animal personality in response to selection.

3.2 Introduction

Understanding the origin and extent of biological variation in natural populations is critical to the study of evolution. Recently, considerable attention has been given to the evolutionary and ecological implications of variation in animal personalities. Broadly defined as repeatable individual differences in behavioural responses over time or across different contexts or both (Gosling 2001; Réale et al. 2007), animal personality has now been documented in over 200 species across many taxa (Carere and Locurto 2011). Individual differences in animal personality influence

life history strategies, species distributions, intra- and interspecific interactions, and population dynamics (reviewed by Wolf and Weissing 2012). As such, describing personality variation and exploring the implications of these traits in natural populations is needed for an understanding of how personality will influence the evolutionary potential of populations, and of species as a whole.

Despite a growing, widespread recognition of the consequences of personality traits, how and why such variation is maintained in natural populations remains unclear (Dingemanse and Réale 2005). Much support has been lent to the life history trade-off hypothesis, wherein variation in personality phenotypes is maintained in a population when a trait is favourable in one context (e.g., reproduction) but maladaptive in another (e.g., conspecific confrontation or predation) (Sih et al. 2004; Wolf et al. 2007; Biro and Stamps 2008). Still, personality variation may be maintained by sexual selection (Schuett et al. 2010), frequency-dependent mechanisms (Dall et al. 2004), or through spatio-temporal fluctuations resulting from environmental flux (Sloan Wilson 1998; Dingemanse et al. 2004). Empirical studies investigating the relationship between life history traits and the personality of wild animals are thus fundamental to unraveling the processes shaping personality trait variation (Biro and Stamps 2008; Smith and Blumstein 2008). Particularly interesting, yet limited, are studies exploring fitness consequences of personality in long-lived species (e.g., Delgado and Penteriani 2008; Patrick et al. 2013; Campioni et al. 2015), as these animals experience varying life-history strategies with certain phenotypic traits (e.g., traits associated with reproductive effort) influenced by aging and senescence (Turbill and Ruf 2010). For example, Patrick and Weimerskirch (2014) investigated the influence of personality on senescence and reproductive success in a long-lived sea bird, the wandering albatross (*Diomedea exulans*), and found a marked decline in reproductive performance associated with personality in males of increasing age.

Réale et al. (2007) has provided a framework for investigating personality by describing five categories of personality traits that includes: activity, boldness, exploration, aggression, and sociability. Of the personality categories described, phenotypes measured along the shy-bold continuum have been investigated extensively and have contributed much to what is known about animal personality across species (Sih et al. 2004); thus, making the shy-bold continuum favourable for comparative study (but see Carter et al. 2013; Beekman and Jordan 2017). Boldness is generally defined as an individual's response to a potentially risky situation (Réale et al. 2007), and has been shown to be highly repeatable and heritable in mammals, birds, fish, herpetofauna, and invertebrates

(Réale et al. 2007; Mayer et al. 2016). In their meta-analysis investigating the fitness consequences of personality, Smith and Blumstein (2008) found boldness to have a positive effect on reproductive success. For example, in female bighorn sheep (*Ovis canadensis)* bolder females had a higher weaning success and began reproducing at an earlier age than shyer counterparts (Réale et al. 2000). However, Bridger et al. (2015) found bolder male hermit crabs (*Pagurus bernhardus*) to have lower fecundity than risk-averse individuals, suggesting that associations of fitness-relevant behaviour with personality may be species-specific and/or influenced by environmental conditions under which the trait is measured.

A long-term study on individually marked grey seals (*Halichoerus grypus*) provides an opportunity in which to investigate the influence of personality on maternal reproductive success. Grey seals in the northwest Atlantic haul out on land or ice annually to breed (Mansfield and Beck 1977). During these discrete reproductive seasons, lasting approximately 2-3 months (December to early February) (Boness and James 1979), grey seals haul out in aggregations forming breeding colonies (Mansfield and Beck 1977). As capital breeders, grey seals rely on stored blubber reserves for energetic demands during time ashore (Boness and James 1979; Iverson et al. 1993). Foraging resumes upon the cessation of breeding when grey seals return to the ocean, where a majority of their time is spent outside of the breeding season (Breed et al. 2009). Females give birth to a single pup each year beginning at 4-6 years of age and can continue to reproduce into their 30s and early 40s (Bowen et al. 2006). Males provide no parental care, but defend females from other males to gain access to matings toward the end of the lactation period (Boness and James 1979; Lidgard et al. 2005). Maternal investment is high, yet brief with an average lactation period lasting 16-18 days (Boness and James 1979; Iverson et al. 1993), after which time the female abruptly weans her pup and returns to the sea. The brief lactation period is a vital one during which females must transfer enough stored energy (Iverson et al. 1993; Mellish et al. 1999) to sustain her pup through a post-weaning fast of 3 weeks before it undertakes independent foraging (Noren et al. 2008). The condition of pups at weaning has thus been shown to be a strong indicator of early pup survival (Hall et al. 2001), with longer and heavier pups having better survival probabilities (Bowen et al. 2015).

A seal pup's survival is dependent not only on adequate milk energy, but also on maternal protection from conspecifics (Kovacs 1987). As is true in other phocid species that breed in densely populated colonies, maternal boldness and aggression may serve as an effective means to increase

the probability of offspring survival (see, for example, Harcourt 1992). Previous work investigating maternal behaviour of grey seals has shown consistent individual differences in a female's propensity to check on her offspring when exposed to a disturbance (Twiss et al. 2012). The high repeatability reported in Twiss et al. (2012) indicates consistent inter-individual variation among females in a component of parental care, with reactive females (i.e., higher behavioural flexibility and lower levels of aggression) found to exhibit higher offspring-checking rates during disturbances. In addition, reactive females were found to have greater variation in pup growth rates (Twiss et al. 2012). While the authors were limited by sample size, their study prompts further investigation into the ecological and reproductive consequences of animal personality in female grey seals.

In this study, we aim to determine if a personality signal exists in a population of grey seals for which long-term life history and demographic data are available. Over nine breeding seasons, we measure individual behavioural responses of female grey seals along the shy-bold continuum, with repeated measures obtained between and within years. We test for the effects of environmental and biological sources of variation on boldness and subsequently estimate the repeatability of boldness. We then assess the influence of boldness on a component of female fitness, offspring weaning mass. If grey seal females on Sable Island display repeatable boldness scores within and between years, we expect offspring weaning mass to be influenced by a female's shy-bold phenotype, as individual variation in maternal behaviour has been shown to influence reproductive success and early offspring survival (e.g., Christenson and Le Boeuf 1978). Assuming boldness increases reproductive success (Smith and Blumstein 2008), we predict boldness will have a positive effect on pup weaning mass in female grey seals, with bolder females weaning heavier offspring on average than shyer counterparts.

3.3 Methods

3.3.1 Study site and population

The study was conducted on Sable Island (44°55'N, 60°00'W), located approximately 300 km off the coast of Nova Scotia, Canada. Sable Island is a sandbar roughly 42 km long and 1.5 km at the widest point. It is characterized by a series of shifting sand dunes with nearly 40% of the island's surface covered in vegetation, predominantly marram grasslands (Catling et al. 1984). The

island currently supports the world's largest breeding colony of grey seals (Bowen et al. 2007), with an estimated 394,000 individuals in the year 2014 (Hammill et al. 2014).

Grey seals are a highly philopatric species (Pomeroy et al. 1994) that haul out on Sable Island in December and January each year to give birth and to mate. This colony has been the focus of long-term demographic research dating back to the 1960s. Longitudinal data (i.e., individual animals observed more than once) were collected during annual censuses of previously marked females. These females were individually and permanently marked with a three- or four- character hot-iron brand applied to the lower back shortly after they weaned, and thus were of known age. Females used in this study were marked in 1973, 1974, 1985-87, 1989, and 1998-2002 (Table B.1). The presence of a study female in the breeding colony was determined during daily surveys throughout the colony and/or during weekly, whole-island censuses of all marked individuals as described in Bowen et al. (2006). Upon locating study females, the mother-pup pair was monitored on a daily basis until weaning, which is marked by the abrupt departure of the female from the colony. Pups were given a uniquely numbered tag (www.daltontags.co.uk) applied to the webbing of the hind-flipper prior to weaning. Pup sex, breeding habitat type, and a boldness score (as described below) were recorded at the time of tagging.

3.3.2 Measurement of boldness scores

Boldness was measured for a total of 469 branded females during nine consecutive breeding seasons (2008 to 2016). In this study, boldness (i.e., female defense of pup) is determined according to the response of the female to human approach and handling of her pup. Together, three researchers slowly approached females from a distance of ~5m. One researcher tagged the pup, while the other two researchers protected the tagger from the focal female and neighbouring adults. The approach typically lasted about a minute. Response to a human approach has been successfully demonstrated in other systems as a means to assess boldness (e.g., Carter et al. 2012; Patrick et al. 2013). Here, boldness was scored on an ordinal scale from one to three: 1= shy, female moves away (female quickly moves at least 1 meter away from researchers and pup); 2 = intermediate, female stays nearby (within 1 meter) and shows no acts of boldness; and 3 = bold, female does not move away and makes abrupt movements in the direction of researchers, vocalizes loudly, lunges towards researchers, displays an open mouth threat, and/or attempts to bite (Table B.2). Not all females successfully reproduced each year and, in some cases, pups died before boldness could be scored.

Therefore, observation numbers varied between individuals. Over a 9-year period, 2504 observations were made for between year repeatability measures. Boldness scores were obtained within a single year on 35 females between 2009 and 2016 (105 total observations). On average, females were tested 5.5 ± 0.09 SE and 3.0 ± 0.30 SE times per female for between and within year sampling efforts, respectively.

The use of a blinded protocol was not possible or appropriate, as our study involved recording behaviour of focal, free-ranging females in the field. However, to avoid bias, the group of three researchers (described above) reached a general consensus as to what boldness score a female received prior to departing the location where the female was tested and her pup was tagged. Behavioural tests were led and performed by the same researchers (WDB, DCL, SLCL, CD) each year.

3.3.3 Measurement of reproductive success

The weaning mass of pups was used as a proxy of female reproductive performance. Once a female returned to the sea following the lactation period, signifying termination of maternal care (Boness and James 1979), her pup was identified by its hind-flipper tag and weighed to the nearest 0.5 kg. Pup mass at weaning is a reliable estimate of maternal energy expenditure and performance in the Sable Island population of grey seals (Iverson et al. 1993; Mellish et al. 1999), with survival being positively related to mass at weaning (Hall et al. 2001; Bowen et al. 2015). In addition to pup weaning mass, we attempted to evaluate weaning success as a second component of female reproductive performance. As all maternal care ceases upon weaning, weaning success can be determined if the weaned pup had a developmental stage indicative of possible survivorship (pelage stage 3 or higher; Bowen et al. 2003, 2015) (Table B.3). A female was recorded as unsuccessful (0) if she was observed with a dead pup or abandoned her pup at developmental stage 1 or 2, and was deemed successful (1) if her weaned pup was at developmental stage 3, 4, or 5. However, annual weaning success as a measure of reproductive performance was not informative, as nearly all females that received a repeated boldness score successfully weaned a pup. Only 45 out of 2504 observations resulted in failed weaning attempts; thus, weaning success as a proxy of reproductive performance was not considered in further analyses.

3.3.4 Characterization of breeding habitat type

Sable Island is defined by various microhabitat features, such as sand dunes, vegetated dunes, sand inland and shoreline areas prone to flooding from tidal influence and storm surges, sand inland and shoreline areas not prone to flooding, and hummocks. We collapsed these microhabitat features to include three habitat categories for analysis: 1 = sand along the shoreline which is unaffected by tidal flooding or sand inland which is unaffected by flooding from storm surge; 2 = vegetated or sand dune habitat; and, 3 = sand along the shoreline which is affected by tidal flooding or sand inland which is prone to flooding from storm surge (Table B.4). While one habitat type is seemingly not better for pupping on Sable Island (WDB, unpublished observation), habitats prone to flooding on the island are deemed as poor-quality habitat (Weitzman et al. 2016), as mother-pup pairs can become separated during flooding events (WDB, unpublished observation). Therefore, categories 1 and 2 can be considered relatively high-quality habitat, yet differentiated by vegetative and fine-scale topographical features. Category 3 was scored as low quality due to the preponderance of flooding from storm surges and tidal influence.

3.3.5 Factors affecting boldness

We tested the effect of four factors (maternal age, weaning date, habitat, observation number) on the response variable, boldness score, by fitting ordinal mixed models using the R package: ordinal (Christensen 2015). All statistical analyses were performed using R 3.3.1 (R Core Team 2016). We included maternal age as a continuous variable to test for an effect of age on boldness. Parity, the number of times an individual was seen pregnant or with a pup, was known for the majority of study females born after 1985. Screening covariates for collinearity demonstrated that parity and age were highly correlated (Pearson's correlation coefficient = 0.96) and, thus, only age was included in models.

Females hauling out to give birth later in the breeding season may experience more harassment from males looking for mating opportunities (Boness et al. 1995). Date of parturition may then affect female boldness with the expectation that individuals with later parturition dates will be bolder than females that give birth earlier in the season. In our dataset, exact parturition date was known for fewer females than those with a weaning date. Therefore, we adopted the approach of Weitzman et al. (2016) to use "weaning date" of females found by calculating the number of days between approximately the first day of the breeding season (i.e., November 30th) and the day

of weaning. Using this method, we determined that "parturition date" (days between November 30th and birth) was highly correlated with weaning date (Pearson's r = 0.93). As such, weaning date was used as a proxy for birth date, increasing the sample size for analyses. To limit the influence of weaning date as a covariate, weaning date was mean centered and scaled to zero with a standard deviation of 1.

The distribution of grey seals on Sable Island has been documented as being less dense in dunes habitat than shoreline areas (Ambs et al. 1999). As such, breeding habitat type was included in models with the expectation that females occupying sand shoreline and inland areas will be bolder than females in less dense habitat (i.e., dunes) given the preponderance of conspecific interaction in more densely occupied space. Alternatively, females occupying higher quality pupping and breeding habitats (categories 1 and 2) may be bolder than females in lower quality areas (category 3), as bolder females may outcompete shyer counterparts for higher-quality resource use. Habitat was fitted as a 3-level factor in models.

Finally, to test that a female's boldness score was not affected by the number of times she was assessed in regard to habituation or altered reaction in response to the approach of researchers (Stamps et al. 2012), observation number was fitted as an ordered factor. Researcher was not corrected for in models, as observations were made by the same researchers each year following a standardized approach of animals and not expected to create bias.

Year was included as a random effect to account for inter-annual environmental variation, which might influence a female's response to researcher approach. Female identity was also included as a random effect in all models. Visual inspection from models did not uncover unexpected deviations or trends. Likelihood ratio tests were used to test the significance of fixed effects by comparing models with and without the term of interest.

3.3.6 Estimating repeatability

We tested for repeatability to estimate the degree of differentiation between females relative to the total phenotypic variation found in the Sable Island population (Boake 1989). Significant sources of variation (maternal age and breeding habitat type) were retained for estimating repeatability using generalized, linear-mixed models (GLMM) set in a Bayesian framework with an ordered data structure in the R package: MCMCglmm (Hadfield 2010) (see Patrick et al. 2013). We defined parameter-expanded priors (V = 1, ν = 1000, $\alpha\mu$ = 0, and αV = 1) for each random effect

and fixed the residual variance to 1 (de Villemereuil et al. 2013). Posterior distributions were sampled every 200 iterations for 1,000,000 total iterations, after an initial burn-in period of 20,000 iterations. Plots of the posterior distributions of the intercept and variance components were visually inspected to determine validity of algorithms and that models converged. We further evaluated autocorrelation among samples, and assessed significance of fixed and random effects by checking that confidence intervals did not overlap zero and by comparing models according to deviance information criterion (DIC), respectively. Inverse gamma priors (V = 1 and v = 0.002) were subsequently specified to ensure that priors initially defined did not have much of an effect on the overall analysis and results obtained (Tables B.5-B.7). To obtain estimates for within year repeatability, posterior distributions were sampled every 1000 iterations, after a burn-in of 30,000 iterations, for 1,300,000 total iterations.

Only females with at least two observations were included in between (n = 458 females) and within year (n = 35 females) repeatability analyses. Variance components were retrieved from the Bayesian GLMM described above by calculating the modes of the posterior distributions, with the probit distribution variance added to the total population variance. Repeatability estimates of boldness were found by dividing the individual (identity) variance by the total population variance (Nakagawa and Schielzeth 2010). We extracted 95% credibility intervals for all repeatability estimates.

3.3.7 Reproductive consequences of personality

We used *lme4* (Bates et al. 2015) to perform GLMM analyses to assess the relationship between a component of maternal performance (weaning mass of offspring) and boldness. We considered the effect of six sources of variation on weaning mass: boldness, maternal age, offspring sex, breeding habitat, breeding success of the female in the previous year, and weaning date. As the number of reproductive events increases and senescence occurs with increasing longevity, components of fitness are expected to be age-related in grey seals (Bowen et al. 2006). Therefore, maternal age was included as a 5-level grouped fixed effect, with age binned into 5-year intervals. However, the fifth group included all females aged 26 and older to account for the reduced sample size of this age bracket. Age classes were defined by: group 1 = 6-10 years (young, growing, and less experienced); group 2 = 11-15 years (young and growing); group 3 = 16-20 years (prime and slow growth); group 4 = 21-25 years (prime and slow growth); and group 5 = 26- 43 years (old and

slow growth) (see Bowen et al. 2006). Offspring sex was fitted as a 2-level fixed factor, with evidence that male pups are typically heavier at weaning than female pups (Bowen et al. 2005; Weitzman et al. 2016). Pups weaned in higher quality habitats (i.e., sand inland or along the shoreline above tidal influence and dune habitat) on Sable Island have been found to weigh slightly more than those weaned in areas prone to flooding (Weitzman et al. 2016) and, thus, habitat was included in models as a 3-level fixed factor. Breeding success in the previous year was included as a 2-level fixed factor (successful or not successful) to account for energy expended in the prior years pup (e.g., Pomeroy et al. 1999). Lastly, weaning date was included as a re-scaled, mean-centered numeric variable with standard deviation of 1.

Pup weaning mass was fitted as the response variable, with study year and individual included in the models as random effects. Likelihood ratio tests were used to assess significance of the effect in question ($P < 0.05$). We further assessed model fit by estimating the coefficient of determination (R^2) for fixed effects alone (marginal R^2) and for both fixed and random effects (conditional R^2) (Nakagawa and Schielzeth 2013). Mean values are provided with standard error estimates throughout.

3.4 Results

3.4.1 Boldness

Boldness scores (n = 2504 observations) for between year repeatability analyses were determined for 458 females ranging in age from 6 to 43 years, and collected over a nine-year period. Mean boldness of females across years was 1.8 ± 0.01 (median boldness score = 2). Age was an important factor affecting boldness (χ^2= 15.01, df = 1; $P < 0.001$; Table 3.1), where younger females were less bold than older individuals (Figure 3.1a). Breeding habitat type also influenced boldness, such that females in dunes habitat were slightly bolder ($\chi^2 = 9.87$, df = 2; $P < 0.01$; estimate = 0.41 ± 0.13) than individuals in either shoreline or inland sand areas, regardless of the flooding tendency (Table 3.1, Figure 3.1b). Weaning date ($\chi^2 = 1.83$, df = 1; $P = 0.18$) and observation number ($\chi^2 = 13.45$, df = 8; $P = 0.1$) did not have significant influences on boldness. Only age and habitat were retained as fixed effects in the Bayesian GLMMs used to estimate repeatability of boldness. Between year repeatability of boldness in the Sable Island population was high (R: 0.61 [CI: 0.57-0.66]; Table 3.2, see Table B.5-B.6 for full model details).

Within year repeated measures (n = 104 observations) were obtained for 35 females with a mean boldness score of 2.1 ± 0.06 (median boldness score = 2). Again, age and habitat were retained as covariates for the Bayesian GLMM analysis. The within year repeatability estimate was high (R: 0.82 [CI: 0.54-0.92]; Table 3.2, see Table B.7 for full model details).

3.4.2 Relationship between boldness and reproductive performance

Weaning masses were not available for all observations for which boldness scores were attained, yielding a reduced dataset of 2020 observations, but on 469 females aged 6-41 years. The average offspring weaning masses for shy, intermediate, and bold phenotypes were 52.6 ± 0.27 kg, 53.1 ± 0.22 kg, and 54.6 ± 0.55 kg, respectively (Figure 3.2). However, boldness, as a main effect, was not significant in explaining weaning mass of pups ($\chi^2 = 2.82$, df = 2; $P = 0.24$). Sources of variation on weaning mass came from differences in maternal age ($\chi^2 = 94.9$, df = 4; $P < 0.001$), pup sex ($\chi^2 = 79.85$, df = 1; $P < 0.001$), breeding habitat type ($\chi^2 = 11.77$, df = 2; $P < 0.01$), and weaning date ($\chi^2 = 4.29$, df = 1; $P = 0.038$) (Table 3.3). The fixed and random effects of the preferred model explained 53% of the variance ($R^2_{GLMM(c)} = 0.53$), while the fixed effects (maternal age, pup sex, weaning date, habitat) of the preferred model explained 6.4% of the variance ($R^2_{GLMM(m)} = 0.064$).

Maternal age influenced weaning mass of pups, such that females belonging to age groups 3 and 4 (both characterized by females in their prime) weaned pups that were, on average 4.3 and 6.2% heavier than pups produced by young and less experienced females, respectively (Table 3.3, Figure 3.2). Pup sex significantly influenced weaning mass with male offspring weighing approximately 2.3 kg more than female offspring. Pups with later weaning dates weighed slightly less than those successfully weaned earlier in the breeding season. Further, females breeding in dunes and floodable habitat produced lighter pups than in non-floodable sand shoreline and inland areas (Table 3.3).

A second model with the inclusion of an interaction term between age and boldness also had strong support (Table B.8). There was a significant effect of variation from the interaction between boldness and age ($\chi^2 = 17.62$, df = 8; $P = 0.024$), such that females aged 11-15 years with the bold phenotype (score 3) produced significantly heavier pups (5.4% heavier; Table 3.3; Figure 3.2; Figure B.1). The confidence intervals of all other interactions between age group and boldness overlapped zero indicating a lack of statistical significance (see Table B.9 for full model details).

Again, pup sex ($\chi^2 = 77.44$, df = 1; $P < 0.001$) and weaning day ($\chi^2 = 4.41$, df = 1; $P = 0.036$) significantly influenced offspring weaning mass. There was no effect of breeding success in the previous year ($\chi^2 = 1.37$, df = 1; $P = 0.24$), or from the interaction between boldness and habitat ($\chi^2 = 7.99$, df = 4; $P = 0.092$; Table B.10). We focus our discussion on results from the more parsimonious, and, thus, preferred model (i.e., boldness assessed as a main effect).

3.5 Discussion

In this study, we showed there were consistent individual differences in boldness of female grey seals exposed to a potentially risky situation. As such, we have described the existence of a personality signal along the shy-bold continuum, measured here as a female's tendency to protect offspring. Boldness varied with maternal age, such that younger females (~6-10 years of age) were shyer than older and more experienced females, and thus provides support for the life-history trade-off hypothesis that predicts younger individuals will be less bold as they have higher future reproductive potential (Wolf et al. 2007). Pups produced by bolder females were on average heavier than pups of shy females, but after accounting for maternal age, pup sex, and breeding habitat type, boldness alone did not influence weaning mass. As shown in previous studies on grey seals, weaning mass of pups in our study was strongly influenced by maternal age (Bowen et al. 2006), offspring sex (Bowen et al. 2006, Weitzman et al. 2016) and habitat. As boldness is correlated with maternal age and habitat, it may be difficult to distinguish the effect of personality on weaning mass from these two factors.

Recent articles in the animal personality literature call for researchers to use a multi-trait, multi-test approach when assessing personality traits in wild populations to provide validation that the trait of interest is the actual trait being measured (Carter et al. 2013; Roche et al. 2016). While prior research constraints prevented using such an approach in the current study, we are confident we assayed personality in a manner that is ecologically relevant to the Sable Island grey seal system. In grey seal breeding colonies, injuries to young pups by non-maternal females are inflicted in direct acts of aggression (Coulson and Hickling 1964), while other sources of injury to pups may arise from males during male-male conflict, breeding attempts, and acts of aggression toward pups (WDB unpublished observations; Bishop et al. 2016). Pups with exposed injuries are susceptible to bacterial infection, a common cause of mortality among individuals of this age group (Baker 1984). Females frequently take risks to ensure protection of her offspring by behaving aggressively

towards female and male conspecifics and/or by directly placing herself between the source of threat and her pup (Boness et al. 1982). Boldness is generally described as a reflection of an animal's reaction to a threat (Sloan Wilson et al. 1994), and as such, the test for boldness used in this study elicited a natural response of offspring protection, while further mirroring tests that have successfully demonstrated boldness in other studies and systems (Carter et al. 2012; Patrick et al. 2013).

We found boldness to be highly repeatable between and within years in female grey seals, suggesting that not only are shy-bold phenotypes maintained consistently across successive years, but also throughout different stages of lactation. Within year repeatability was higher than between year estimates; a finding consistent with Bell et al. (2009) who report in their meta-analysis that repeatability is typically higher when measurements are made closer together in time. Further, repeatability estimates in this study are comparable to those in previous grey seal studies that investigated individual consistency of various behaviours including male tenure duration in defense of females (Lidgard et al. 2012), male alertness (Twiss and Franklin 2010), and female pup-checking behaviour (Twiss et al. 2012).

Critics of animal personality contend that the field has generated little novel insight owing in part to, and among other flaws, terminological inconsistencies and confusion surrounding definitions of such terms (Beekman and Jordan 2017; Jungwirth et al. 2017). For instance, Beekman and Jordan (2017) raise the concern that personality is often used in the literature to describe behavioural flexibility and that it is subject to temporal change despite "consistency" being a key component of animal personality. Nevertheless, experiential factors may influence the development of behavioural phenotypes (Stamps and Groothuis 2010). In fact, how stable behavioural differences are over ontogeny remains an intriguing question in animal personality research (Wilson and Krause 2012; Sih 2017). As of now, we have shown that boldness varies with maternal age in grey seals, which may be a product of selection on boldness and survival, or better explained by trade-offs.

Life-history theoretical models predict individuals with high future reproductive potential should alter their behaviour to be more risk-averse (i.e., less bold) when confronted or threatened with danger, whereas those with low reproductive potential should theoretically invest more in current reproductive efforts (Wolf et al. 2007; Biro and Stamps 2008). It stands to reason that young individuals with high future fitness expectations have more to lose by behaving boldly when placed

in a risky situation (Wolf et al. 2007). We found younger and less experienced females were on average less bold than older, more experienced females suggesting a trade-off between growth and fecundity (Figure 3.1a). Female grey seals have exceptionally long reproductive life spans, reaching sexual maturity at 4-6 years of age and continuing to reproduce into their late 30's and early 40's (Bowen et al. 2006). Therefore, grey seal females would benefit from being more risk-averse at an earlier age given their high future reproductive potential. While grey seals experience a decline in maternal performance (e.g., capacity to deliver milk energy) from physiological factors related to aging (Bowen et al. 2006), we found no evidence to suggest that senescence affects boldness in older grey seal females, as was found in another long-lived marine species (Patrick et al. 2014). In fact, the oldest grey seal females seemingly continue to attend pups throughout the entire lactation period (Bowen et al. 2006).

Alternatively, differences in boldness between age groups of females may be explained by size-related differences. While we were unable to assess the effect of maternal size in the current study, postpartum body mass has been shown to vary with age in grey seal females, such that mass at parturition continues to increase into an individual's middle teens before stabilizing and remaining relatively constant throughout adulthood (Bowen et al. 2006; Lang et al. 2009). Evidence exists in some animal systems that larger individuals may be better able to afford the risk of being bolder than smaller counterparts (Maillet et al. 2015; Mayer et al. 2016). For instance, Mayer et al. (2016) discovered that larger offspring, in the absence of experience, behaved more boldly than smaller counterparts. Yet, other studies suggest that it is boldness that determines body mass (Brown et al. 2007). In a review, Biro and Stamps (2008) found support that variation in boldness, aggression, and activity seems to be positively associated with food acquisition and intake rates (i.e., growth). Whether a mechanism between size, and/or intake rates, and personality exists in the grey seal remains to be seen.

Sable Island's topography is dynamic and is characterized by different microhabitat features (i.e., beaches, sand inland areas, dunes, vegetated areas) that offer nearly unrestricted access to breeding sites for seals. We found females in dunes habitat were bolder than females in sand inland and shoreline areas, regardless of flooding tendency (Figure 3.1b). However, the observed effect of habitat type on boldness is biologically uncertain here. The influence of habitat on personality may be the result of local density-dependent and social activity factors at whelping sites, rather than breeding habitat type per se. It is understood that females returning to give birth and to breed on

Sable Island rarely exhibit site fidelity (Weitzman et al. 2016), but it is not clear what cues individuals use in selecting certain breeding sites on the island. Grey seals are generally a gregarious species, such that females often aggregate during the breeding season (Boness and James 1979). Still, females of a particular personality type may preferentially select less-dense sites in an effort to minimize the costs of social conflict during the lactation period. That is, personality type may be driving the adoption of a specific social niche among individuals (Bergmüller and Taborsky 2010). The consistent use of breeding habitat sites, as well as the effect of local density and activity on female grey seal behaviour warrants further investigation.

While we did not find a significant association between offspring weaning mass and boldness as a main effect in the preferred model, females with the boldest phenotype produced pups with an average of ~2 kg more than pups of the shyest females and younger females that were bolder produced significantly heavier pups than shyer counterparts (Figure 3.2). The apparent advantage of boldness on pup weaning mass may decline with maternal age, an interaction worthy of further exploration as a new subset of branded females return to Sable Island as first-time breeders. An overtly shy female may not allocate as much resources to her pup if she is easily disturbed and flees often, interrupting essential suckling bouts in the process (see Boness et al. 1995). Fat deposition accounts for the majority of offspring mass gained during lactation and represents approximately 92% of energy stored at weaning (Mellish et al. 1999). It seems likely the difference in weaning mass observed in the current study is in the form of fat stored as blubber, which serves an insulative purpose and also as an energy reserve maintaining metabolic demands throughout the post-weaning fast of phocid pups (Worthy and Lavigne 1987; Noren et al. 2003). The observed weaning mass difference between pups of females with different personality phenotypes could potentially serve to sustain pups longer during fasting, and thus permit further physiological development (e.g., increased oxygen store capacity) (Noren et al. 2008). Noren et al. (2008) found that leaner pups at weaning terminate their post-weaning fast prematurely, which may then have a direct influence on early diving proficiency upon commencement of foraging. Bowen et al. (2015) further showed a non-linear positive relationship between offspring weaning mass and survival, with the probability of survival roughly doubling between 40 and 50 kg. The ~2-kg mass difference observed for pups of bold females versus shy females, though not statistically significant, may be biologically meaningful by conferring an early survival advantage for larger offspring.

Grey seal female size and milk energy output are among the best predictors of pup weaning mass (Iverson et al. 1993; Mellish et al. 1999). Lang et al. (2009) further discovered consistent individual differences in maternal performance traits, such as lactation duration, milk energy content, and daily milk energy output. Since experienced females within peak breeding age (~16-26 years) are larger (Bowen et al. 2006; Lang et al. 2009), it may be possible that personality of females is associated with maternal size, as hypothesized earlier, and thus influences other maternal reproductive performance traits not yet examined. Nevertheless, sources of variation found to influence weaning mass reported in this study corroborate findings of others linking weaning mass differences with maternal age (Bowen et al. 2006), pup sex (Bowen et al. 2006; Weitzman et al. 2016), weaning date (Boness et al. 1995), and, although representing a slight variation here, habitat (Weitzman et al. 2016).

The results of our study demonstrate that female grey seals show consistent individual differences along the shy-bold continuum of animal personality. The strength of differentiation detected in grey seals suggests that boldness may have a genetic basis (but see Dohm 2002), as has been found in other studies (Réale et al. 2007). While boldness alone was not significantly linked to pup weaning mass, relationships between boldness and other life history traits should be examined. Insofar, this study is the first step in describing the repeatability of boldness in an under-represented group of wild marine mammals in the animal personality literature, and further sets the stage for the investigation into the proximate and ultimate factors influencing personality in an ecologically important marine predator.

Table 3.1: Parameter estimates for the preferred ordinal mixed model (clmm) assessing biological and environmental sources of variation on boldness in female grey seals on Sable Island, Nova Scotia (Canada).

Coefficients	Estimate	SE	*z*	*P*
Age	0.096	0.017	5.69	< 0.001
Dunes	0.41	0.13	3.08	< 0.01
Sand (Flood)	0.24	0.15	1.59	0.11
Threshold				
1|2	0.61	0.31	1.95	
2|3	5.50	0.36	15.2	

In the threshold coefficients, 1 represents the shy phenotype (flee), 2 the intermediate phenotype, and 3 represents the extreme boldness phenotype. Individual identity and year were included as random effects

Table 3.2: Sample sizes, mean boldness scores (± SE), and among-individual and among-year variances (95% CI) for estimation of between and within year repeatability estimates (*r*) of boldness for the Sable Island population of female grey seals.

Trait	Random effects	N_{obs}; (N_{bold} 1, 2, 3)	Mean (SE)	V_{ID} (CI)	V_{Year} (CI)	*r* (CI)
Boldness (betwn yr.)	Year = 2008-2016 ID = 458 females	N_{obs} = 2504 (889, 1340, 375)	1.8 (0.01)	3.21 (2.69-3.93)	0.0008 (0-0.091)	0.61 (0.57-0.66)
Boldness (w/n yr.)	Year = 2009-2016 ID = 35 females	N_{obs} = 104 (16, 59, 29)	2.1 (0.06)	9.75 (4.25-19.96)	0.035 (0-4.9)	0.82 (0.54-0.92)

N_{bold} 1, 2, and 3 represent the number of observations of females scored as shy, intermediate, and bold phenotypes, respectively. V_{ID}, V_{Year}, and repeatability were estimated from fitting grouped age and habitat as fixed effects. Residual variances were fixed to 1 for all ordinal data models. See Tables B.5-B.7 for model details.

Table 3.3: Parameter estimates of models generated from generalized linear mixed model (GLMM) analysis investigating the sources of variation on offspring weaning mass in grey seals (N_{obs} = 2020 on 469 females) on Sable Island, Nova Scotia (Canada).

	Model 1					Model 2 (preferred)				
Coefficients	Estimate	SE	*t*	CI lower	CI upper	Estimate	SE	*t*	CI lower	CI upper
(Intercept)	53.23	0.79	66.97	51.65	54.90	53.12	0.75	71.19	51.63	54.71
Age Group 2	1.52	0.57	2.65	0.38	2.65	2.14	0.45	4.79	1.24	3.03
Age Group 3	1.56	0.96	1.63	-0.35	3.47	2.28	0.70	3.26	0.86	3.69
Age Group 4	3.29	0.96	3.48	1.43	5.16	3.28	0.65	5.03	1.99	4.57
Age Group 5	0.62	1.11	0.56	-1.57	2.81	-0.57	0.78	-0.73	-2.14	0.98
Pup Sex (F)	-2.29	0.25	-9.02	-2.79	-1.79	-2.30	0.25	-9.05	-2.80	-1.80
Dunes	-0.96	0.31	-3.14	-1.56	-0.36	-0.95	0.31	-3.08	-1.55	-0.34
Sand (Flood)	-0.83	0.35	-2.37	-1.52	-0.14	-0.84	0.35	-2.37	-1.53	-0.14
Weaning Date	-0.39	0.20	-2.01	-0.78	-0.005	-0.38	0.20	-1.95	-0.77	0.01
Bold 2	-0.26	0.57	-0.45	-1.38	0.87					
Bold 3	0.12	1.16	0.11	-2.14	2.39					
Bold 3 x Age 2	2.87	1.25	2.29	0.41	5.33					

*Females are grouped into 5 age categories (Age 1: $N_{6\text{-}10}$ = 342; Age 2: $N_{11\text{-}15}$ = 558; Age 3: $N_{16\text{-}20}$ = 115; Age 4: $N_{21\text{-}25}$ = 202; Age 5: N_{26+} = 114). See Table B.9 for full model details.

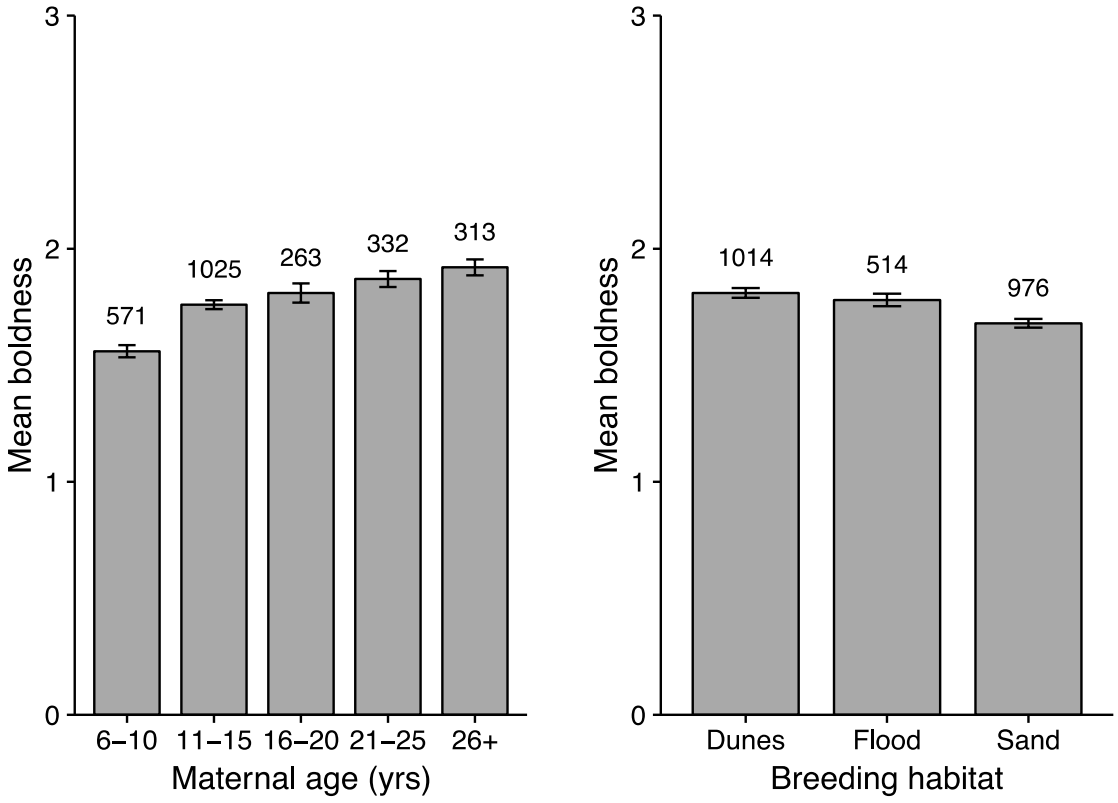

Figure 3.1: The mean boldness scores according to maternal age and habitat type of female grey seals breeding at the Sable Island, Nova Scotia (Canada) grey seal colony. Boldness was scored as: 1 = shy phenotype, 2 = intermediate phenotype, and 3 = bold phenotype. A. The differences of boldness with maternal age. For plotting purposes, females are binned into 5-year age intervals, with females aged 26 years and older lumped into one category. Age was fitted as a continuous variable in analyses investigating sources of variation on boldness. B. The differences of boldness with breeding habitat type, with "Flood" denoting sand inland and shoreline areas prone to tidal influence and flooding and "Sand" representing sand inland and shoreline area that do not flood. The numbers of observations scored per category are provided above bars (N_{total} = 2504 observations). Error bars indicate the standard error around the mean.

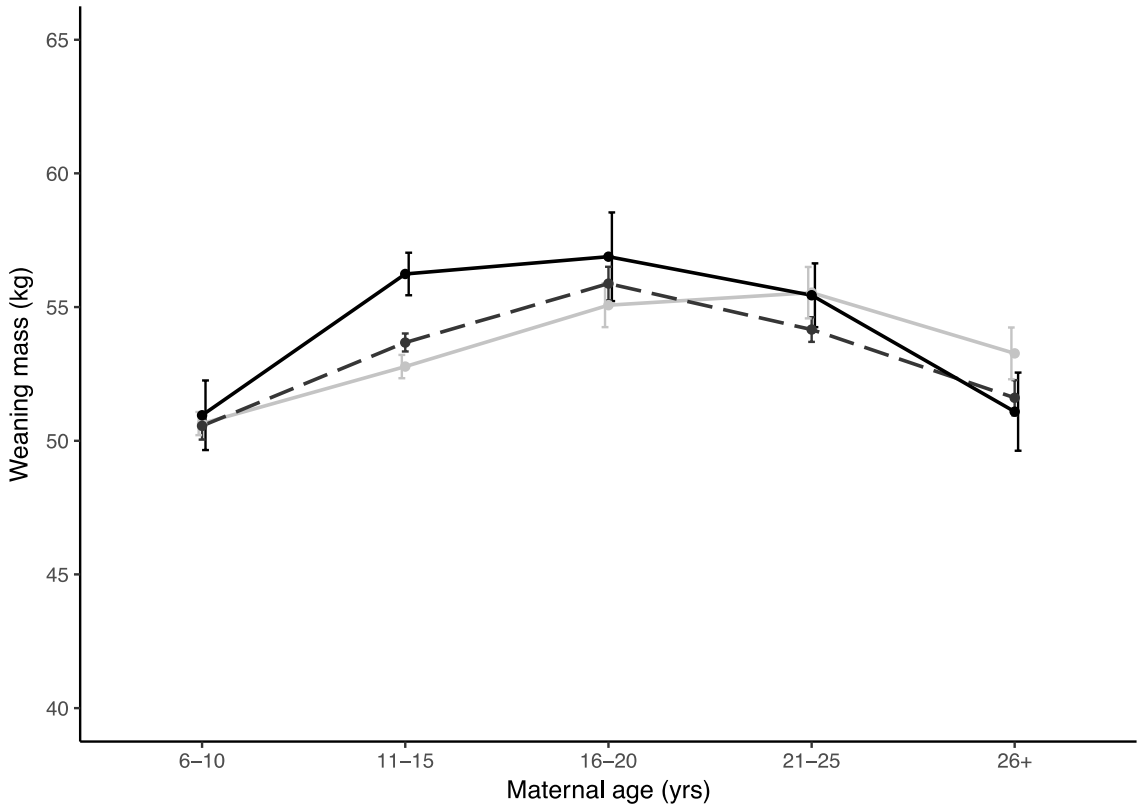

Figure 3.2: The relationship between maternal age and boldness with pup weaning mass (measured to the nearest 0.5kg) in the Sable Island population of female grey seals. The mean weaning mass for each age bracket measured along a shy-bold continuum is plotted with ± standard error. Solid light grey line = shy phenotype; dashed black line = intermediate phenotype; and, solid black line = bold phenotype.

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Appendices

B.1 Additional tables

Table B.1: Breakdown of study females according to cohort (year of birth) and number of observations per cohort.

Cohort	Females	Observations
1973	7	21
1974	7	21
1985	41	179
1986	41	228
1987	23	124
1989	21	95
1998	47	264
1999	88	534
2000	61	369
2001	57	344
2002	65	325

Table B.2: Scores and descriptions of behaviours measured along the shy-bold animal personality continuum as assessed in the field (full behaviour assessment), and the collapsed assessment used for data analysis.

Full Behaviour Assessment			Collapsed Behaviour Assessment		
Score	Behaviour	N	Score	Behaviour	N
1	Flee	110	1	Flee and move away, no boldness	736
2	Move away, no boldness	626	2	Stay nearby, little to no boldness	1072
3	Stay nearby, no boldness	743	3	Moderate to extreme boldness	212
4	Mild boldness	329			
5	Moderate boldness	141			
6	Extreme boldness	71			

Table B.3: Developmental pelage stage of pups used when monitoring mother-pup pairs, and subsequently when assigning weaning success of females.

Stage	Description	Pelage
1	Newborn	Wet from birth; yellowish tint
2	Thin white	White
3	Fat white	White to light grey
4	Molting	Lanugo starting to shed
5	Molted	Lanugo shed

*As adopted from Bowen et al. (2003)

Table B.4: Category and descriptions of breeding habitat type as assessed in the field (full habitat assessment), and the collapsed habitat assessment used for data analysis.

Full Habitat Assessment				Collapsed Habitat Assessment			
Category	Habitat	Tide/Flood	N	Category	Habitat	Tide/Flood	N
1	Sand, shoreline	No	289	1	Sand inland and shoreline	No	801
2	Sand, shoreline	Yes	200	2	Sand or vegetated dunes	No	823
3	Sand, inland	No	477	3	Sand inland and shoreline	Yes	396
4	Sand, inland	No	196				
5	Dunes, vegetated	No	716				
6	Dunes, sand	No	107				
7	Hummocks	No	35				

Table B.5: Estimation of between year repeatability with parameter expanded priors. Variance fixed to 1 for ordinal models.

Random	Post.Mode	CI Lower	CI Upper	Post.Mean	Eff.Samp
ID	3.21	2.69	3.93	3.29	4900
Year	0.0008	0.0000	0.090	0.030	4900
Units	1.00	1.00	1.00	1.00	0
Repeatability					
	0.61	0.57	0.66		

Table B.6: Estimation of between year repeatability with inverse gamma priors. Variance fixed to 1 for ordinal models.

Random	Post.Mode	CI Lower	CI Upper	Post.Mean	Eff.Samp
ID	3.30	2.69	3.98	3.32	4900
Year	0.0017	0.0002	0.067	0.021	3982
Units	1.0	1.0	1.0	1.0	0
Repeatability					
	0.62	0.57	0.66		

Table B.7: Estimation of within year repeatability with parameter expanded priors. Variance fixed to 1 for ordinal models.

Random	Post.Mode	CI Lower	CI Upper	Post.Mean	Eff.Samp
ID	9.75	4.25	19.96	11.26	1246
Year	0.035	0	4.90	1.47	1270
Units	1.0	1.0	1.0	1.0	0
Repeatability					
	0.82	0.54	0.92		

Table B.8: Top three models for assessing sources of variation on a measure of female reproductive performance, pup weaning mass, in grey seal females of Sable Island and the associated Akaike information criterion (AIC) values. Support for the top model provided by lowest AIC, smallest ΔAIC (difference in AIC from top model), and highest AIC weight (*w*).

Model	*K*	AIC	ΔAIC	*w*	LL	$R^2_{GLMM(c)}$	$R^2_{GLMM(m)}$
Age_{Gr} + Sex + Habitat + Wean	12	13112.15	0.00	0.18	-6544.0	0.53	0.064
Age_{Gr} + Sex + Habitat + Wean + Brd	13	13112.24	0.099	0.18	-6543.03	0.53	0.064
Bold:Age_{Gr} + Bold + Age_{Gr} + Sex + Habitat + Wean	22	13112.72	0.569	0.14	-6534.10	0.53	0.071

*Sex = offspring sex; Wean = weaning day; Brd = breeding success in the year prior

Table B.9: Parameter estimates of a highly supported model investigating sources of variation on offspring weaning mass, including an interaction term between boldness and age, in grey seals (N_{obs} = 2020 on 469 females) on Sable Island, Nova Scotia (Canada). Comparisons of top three models are provided in Table B.8.

	Model 1				
Coefficients	Estimate	SE	*t* value	CI lower	CI upper
(Intercept)	53.23	0.79	66.97	51.65	54.90
Age Group 2	1.52	0.57	2.65	0.38	2.65
Age Group 3	1.56	0.96	1.63	-0.35	3.47
Age Group 4	3.29	0.96	3.48	1.43	5.16
Age Group 5	0.62	1.11	0.56	-1.57	2.81
Pup Sex (F)	-2.29	0.25	-9.02	-2.79	-1.79
Dunes	-0.96	0.31	-3.14	-1.56	-0.36
Sand (Flood)	-0.83	0.35	-2.37	-1.52	-0.14
Weaning Date	-0.39	0.20	-2.01	-0.78	-0.005
Bold 2	-0.26	0.57	-0.45	-1.38	0.87
Bold 3	0.12	1.16	0.11	-2.14	2.39
Bold 2 x Age 2	0.64	0.69	0.93	-0.71	2.00
Bold 3 x Age 2	2.87	1.25	2.29	0.41	5.33
Bold 2 x Age 3	1.34	1.07	1.26	-0.75	3.43
Bold 3 x Age 3	0.32	1.65	0.20	-2.92	3.57
Bold 2 x Age 4	0.02	1.03	0.02	-2.00	2.05
Bold 3 x Age 4	0.41	1.78	0.23	-3.08	3.89
Bold 2 x Age 5	-1.18	1.11	-1.06	-3.37	1.00
Bold 3 x Age 5	-2.68	1.87	-1.43	-6.35	0.98

* Females are grouped into 5 age categories (Age 1: $N_{6\text{-}10}$ = 342; Age 2: $N_{11\text{-}15}$ = 558; Age 3: $N_{16\text{-}20}$ = 115; Age 4: $N_{21\text{-}25}$ = 202; Age 5: N_{26+} = 114).

Table B.10: Parameter estimates of the full model from generalized linear mixed model (GLMM) analysis (N_{obs} = 2020 on 469 females) investigating sources of variation on offspring weaning mass in grey seals of Sable Island, Nova Scotia (Canada).

Coefficients	Estimate	SE	*t* value	CI lower	CI upper
(Intercept)	53.16	0.85	62.80	51.48	54.91
Age Group 2	1.63	0.57	2.85	0.50	0.46
Age Group 3	1.71	0.95	1.80	-0.20	3.20
Age Group 4	3.40	0.94	3.60	1.53	2.77
Age Group 5	0.88	1.11	0.79	-1.32	3.62
Pup Sex (F)	-2.26	0.25	-8.91	-2.76	5.26
Dunes	-1.73	0.49	-3.56	-2.69	3.07
Sand (Flood)	-0.76	0.58	-1.32	-1.89	0.37
Wean Date	-0.42	0.20	-2.12	-0.81	-0.03
Breed Success-1	0.36	0.31	1.17	-0.24	0.97
Bold 2	-0.83	0.66	-1.26	-2.13	0.46
Bold 3	0.59	1.33	0.44	-2.03	3.20
Bold 2 x Age 2	0.57	0.69	0.83	-0.78	1.93
Bold 3 x Age 2	2.84	1.25	2.27	0.39	5.31
Bold 2 x Age 3	1.32	1.06	1.24	-0.77	3.40
Bold 3 x Age 3	0.09	1.65	0.05	-3.15	3.33
Bold 2 x Age 4	0.02	1.03	0.02	-2.01	2.04
Bold 3 x Age 4	0.51	1.78	0.29	-2.99	4.00
Bold 2 x Age 5	-1.26	1.11	-1.13	-3.44	0.93
Bold 3 x Age 5	-2.72	1.88	-1.45	-6.40	0.96
Bold 2 x Habitat 2	1.42	0.62	2.28	0.20	2.63
Bold 3 x Habitat 2	-0.10	1.08	-0.09	-2.21	2.01
Bold 2 x Habitat 3	0.17	0.75	0.23	-1.30	1.64
Bold 3 x Habitat 3	-1.76	1.33	-1.32	-4.37	0.85

*Breed success^{-1} denotes a female's breeding success in the previous year, and was fitted as a 2-level factor in models (successful or not) to account for prior reproductive effort. Females are grouped into 5 age categories ($N_{6\text{-}10}$ = 342, $N_{11\text{-}15}$ = 558, $N_{16\text{-}20}$ = 115, $N_{21\text{-}25}$ = 202, N_{26+} = 114).

B.2 Additional figures

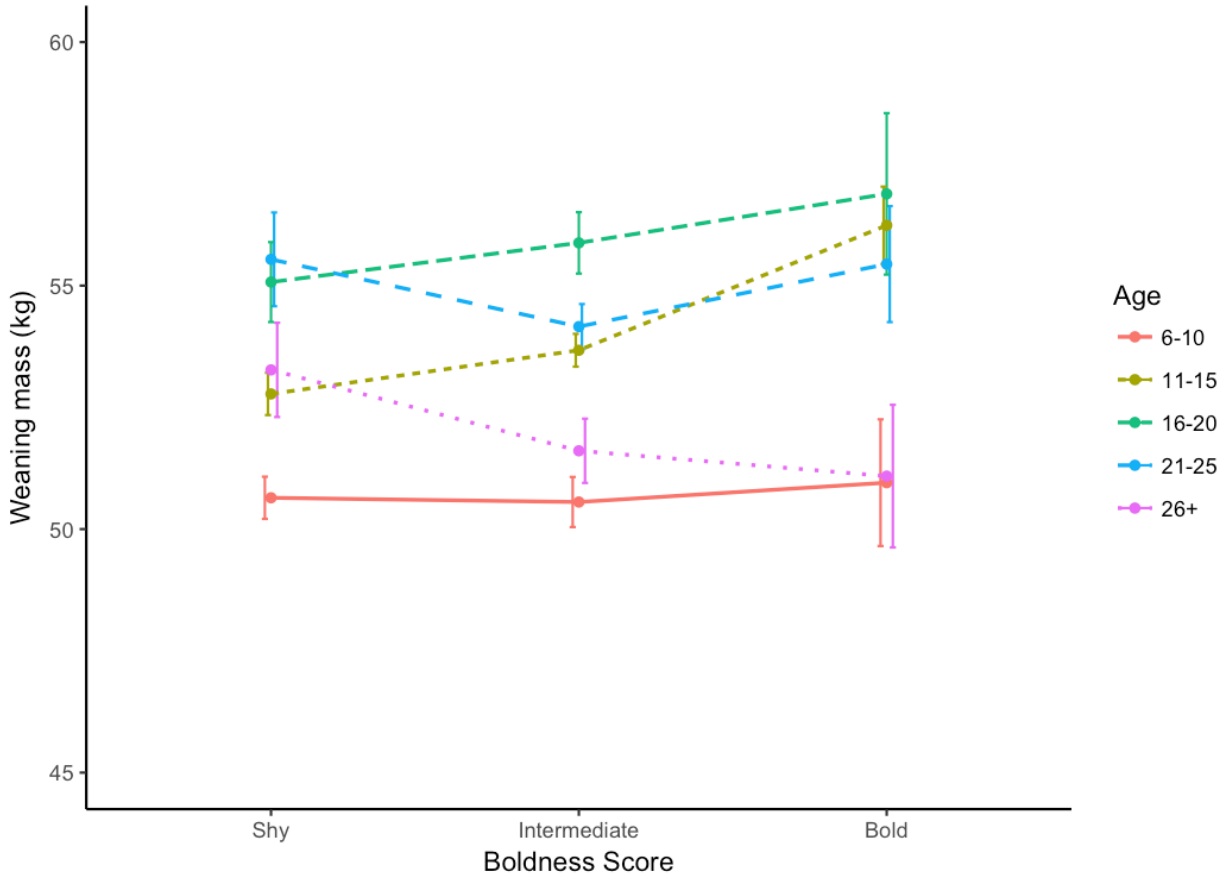

Figure B.1: The relationship between maternal age and boldness with pup weaning mass (measured to the nearest 0.5 kg) in the Sable Island population of female grey seals. The mean weaning mass for each age bracket measured along a shy-bold continuum is plotted with ± standard error.

Chapter 4: Genetic association with boldness and maternal performance in a free-ranging population of grey seals

4.1 Abstract

Individual variation in quantitative traits clearly influence many ecological and evolutionary processes. Moderate to high heritability estimates of personality and life-history traits suggest some level of genetic control over these traits. Yet, we know very little of the underlying genetic architecture of phenotypic variation in the wild. In this study, we used a candidate gene approach to investigate the association of genetic variants with repeated measures of boldness and maternal performance traits (weaning mass and lactation duration) collected over an 11- and 28-year period, respectively, in a free-ranging population of grey seals on Sable Island National Park Reserve, Canada. We isolated and re-sequenced five genes: dopamine receptor D4 (*DRD4*), serotonin transporter (*SERT*), oxytocin receptor (*OXTR*), and melanocortin receptors 1 (*MC1R)* and 5 (*MC5R*). We discovered single nucleotide polymorphisms (SNPs) in each gene; and, after accounting for loci in linkage disequilibrium and filtering due to missing data, we were able to test for genotype-phenotype relationships at seven loci in three genes (*DRD4*, *SERT*, and *MC1R*). We tested for association between these loci and traits of 180 females having extreme shy-bold phenotypes using mixed-effects models. One locus within *SERT* was significantly associated with boldness (effect size = 0.189) and a second locus within *DRD4* with weaning mass (effect size = 0.232). Altogether, genotypes explained 6.52-13.66% of total trait variation. Our study substantiates *SERT* and *DRD4* as important determinants of behaviour, and provides unique insight into the molecular mechanisms underlying maternal performance variation in a marine predator.

4.2 Introduction

Understanding the origin and maintenance of variation in behavioural and life-history traits in the wild remains a challenging area of research (van Oers and Mueller 2010). In a recent meta-analysis on the heritability of behaviour in vertebrate and invertebrate species, Dochtermann et al. (2019) reported an average 24% heritable component for behavioural traits,

comparable to an average 26% for life-history traits (Mousseau and Roff 1987). These heritability estimates suggest an appreciable genetic component underlying phenotypic variation. While estimating heritability is an important starting place in investigating the genetic basis of complex traits (Boake et al. 2002), quantitative genetic approaches cannot determine the genetic architecture (i.e., specific genes or pathways) underlying the heritable component of the variation we observe. Fortunately, advances in molecular genetic methodologies and analytical techniques now facilitate the exploration of genotype-phenotype relationships in natural systems of non-model organisms (Laine and van Oers 2017; Bengston et al. 2018). The goal of this study is to apply these methodologies to study the genetic basis of personality and life-history trait variation in a wild population.

The study of animal personality is a rapidly growing area of research, as evidenced by a large increase in the number of studies published in recent decades (Carere and Maestripieri 2013). Personality, defined as consistent individual differences in behavioural responses across time and contexts (Gosling 2001), influences ecological processes and is subject to selective pressures (Réale et al. 2007; van Oers et al. 2008). With implications for wildlife conservation, management, and animal welfare, personality research plays a key role in our understanding of individual differences in other behavioural categories (e.g., dispersal, reproduction, parental care), as well as evolutionary changes in populations resulting from these differences (McDougall et al. 2006). For instance, individuals of a particular personality type may exhibit more or less care of offspring, potentially affecting reproductive success and productivity. Researchers are now attempting to address fundamental evolutionary questions by examining the proximate mechanisms underlying personality trait (i.e., a quantifiable and specific repeatable characteristic of an individual's behavioural repertoire) variation and the ultimate mechanisms responsible for maintaining variation within and among populations or species (Laine and van Oers 2017; Bubac et al. 2020).

Much research has been undertaken on five major axes of personality: activity, aggression, boldness, exploration, and sociability (Gosling 2001; Réale et al. 2007). These personality traits are often highly correlated forming behavioural syndromes, wherein some individuals are generally more bold, active, and aggressive than their counterparts (Sih et al. 2004). Boldness, widely regarded as an individual's propensity to take risks or an individual's response to a potentially risky situation (Sloan Wilson et al. 1994; Réale et al. 2007), including

interactions with hetero-specifics, has been documented in taxonomically diverse groups such as cephalopod mollusks (Sinn et al. 2008), songbirds (Timm et al. 2018), and ungulates (Réale et al. 2000). Phenotypes along the shy-bold continuum have been associated with variation in survivorship, reproductive success, and dispersal - life-history characteristics with important ecological and evolutionary consequences. For example, in bighorn sheep (*Ovis canadensis*), among-individual variation in boldness was related to female reproductive investment and life-history traits, with bold ewes younger at primiparity and exhibiting higher offspring weaning success than shy females (Réale et al. 2000). Being bold and taking more risks may prove beneficial and adaptive, yet the optimal degree of boldness is ultimately dependent upon the context and/or situation, likely contributing to the maintenance of behavioural variation within populations.

One approach to discovering sources of variation in behavioural and life-history traits is a candidate gene study, wherein a gene of known function is screened for genetic variants to test for association with traits of interest. A favorable option to investigate the genetic basis of complex traits in organisms with limited genomic resources (Fitzpatrick et al. 2005), a candidate gene approach permits tracking allele frequencies of trait-associated genes within and among populations, providing insight into the adaptive selective processes operating on these traits. Currently, a few candidate genes have been associated with the shy-bold continuum in songbirds, primates, and rodents (Laine and van Oers 2017). The dopamine receptor region D4 (*DRD4*) gene has been extensively studied in its association with various personality traits (Savitz and Ramesar 2004). Identified by its relationship with neurological and psychiatric disorders in humans and laboratory organisms (Mitsuyasu et al. 2001; Kluger et al. 2002), *DRD4* has subsequently been associated with similar behavioural effects in domesticated and wild animals. In free-ranging populations of avian and non-human primate species, *DRD4* has been linked with interindividual variation in boldness, novelty seeking, and impulsivity (e.g., Fairbanks et al. 2012 and Riyahi et al. 2015).

Another gene widely studied in relation to personality trait variation is the serotonin transporter gene (*SERT*), a protein coding gene involved in the uptake of serotonin (Savitz and Ramesar 2004). *SERT* has been associated with various behaviours including anxiety, harm avoidance, aggression, and risky behaviour in humans and animals, alike (Lesch et al. 1996; Kim et al. 2006). For instance, Holtmann et al. (2016) found that wild female dunnocks (*Prunella*

modularis) heterozygous at a *SERT* locus engaged in more risky behaviour when approached by researchers than their homozygous counterparts. Still other genes, many with pleiotropic effects, have been identified in explaining a proportion of personality trait variance, such as the oxytocin receptor (*OXTR*) gene and genes of the melanocortin system (Sala et al. 2013; Jacobs et al. 2016). Despite these promising gene-behaviour findings, investigations into the underlying genetic basis of personality in natural systems remains in its infancy (van Oers and Mueller 2010; Laine and van Oers 2017). Further, behavioural-related candidate gene studies focused on wild mammals have been limited in taxonomic breadth to predominantly non-human primates and rodents (Bubac et al. 2020).

Notwithstanding an increased number of personality studies, marine mammal personality research is currently underrepresented. Recent studies on free-ranging pinnipeds highlight the potential role that personality trait variation plays in shaping population dynamics of marine mammals, including detection of relationships between behavioural variation and fitness-related traits and coping strategies within changing and challenging environments (Twiss et al. 2020). Grey seals (*Halichoerus grypus*) provide an excellent system to explore the association between genetics and behaviour, as they exhibit repeatable behavioural variation in the wild (e.g., Lidgard et al. 2012; Twiss et al. 2012; Bubac et al. 2018). The grey seal is a philopatric, colonial-breeding species where individuals haul out on land or ice annually to give birth and to mate (Mansfield and Beck 1977). With long reproductive lifespans (upwards of 35 years) (Bowen et al. 2006), females give birth to a single pup on a nearly annual basis beginning at the age of 4-6 years and provide all parental care during a brief lactation period lasting approximately 16-18 days (Bowen et al. 1992). While grey seals have few natural land-based predators (e.g., canids and predatory/scavenging birds), pups can sustain life-threatening injuries from non-maternal females in aggressive acts and from males in mating attempts, acts of aggression, and during male-male battles for access to mates (Boness et al. 1982; Baker 1984; van Neer et al. 2019). Therefore, by showing an increased level of aggression towards conspecifics in defense of her pup (Boness et al. 1982), a female may improve the survival probability of her offspring in densely populated colonies (McCann 1982). When confronted with a novel object or exposed to a potentially risky situation during the lactation/parental care period, grey seal females respond consistently different from others in frequency of pup-checking rates and protection of offspring from a perceived threat (Twiss et al. 2012 and Bubac et al. 2018, respectively). High

repeatability estimates, serving to set the upper bounds to heritability (but see Dohm 2002), of inter-individual behavioural responses provide evidence of personality signals in local grey seal colonies (Twiss et al. 2012; Bubac et al. 2018), and further indicate that these traits may have a genetic basis.

Patterns have emerged linking personality types with reproductive success in grey seals. Bubac and colleagues (2018) showed that bold females weaned pups that were on average heavier than those of shy moms, providing the pup with a slight early life advantage. In a separate breeding colony of grey seals, Twiss et al. (2012) found that reactive (e.g., behaviourally flexible and generally more docile) females weaned pups with more varied growth rates than proactive individuals. The reasons for these observations remain uncertain. Easily disturbed females may frequently interrupt essential suckling bouts, periods wherein a female must transfer enough milk energy to her pup before weaning occurs (Iverson et al. 1993), and/or exhibit shortened lactation durations. Still yet, a female's genetic makeup may influence reproductive success by directly or indirectly affecting her ability to deliver milk (e.g., daily milk output and milk composition) (Lang et al. 2009). Genes, such as *SERT* and *OXTR*, that have been linked with behavioural variation are also suspected to be associated with parental care phenotypes including offspring responsiveness (Bakermans-Kranenburg and IJzendoorn 2008) and other reproductive parameters (Timm et al. 2018).

In this study, we used a candidate gene approach to explore the relationships between genetic variants and boldness and maternal performance under natural conditions in grey seals. Using a longitudinal database (11 and 28 years for boldness and maternal performance, respectively) and archived tissue samples from the Sable Island National Park Reserve breeding colony of grey seals, our primary objectives were to: a) determine whether seals in our study population show DNA sequence polymorphisms in the *DRD4*, *SERT*, *OXTR*, and melanocortin receptors 1 (*MC1R)* and 5 (*MC5R*) genes, genes previously related with behavioural variation; b) test the hypothesis that individual differences in boldness and life-history traits (pup weaning mass (PWM) and lactation duration) are affected by genetic polymorphisms in the aforementioned genes; and, c) extend our understanding of the relative importance of these candidate genes to phenotypic variation in a wider range of natural systems.

4.3 Methods

4.3.1 Study site

Our field study was performed on Sable Island National Park Reserve (hereafter Sable Island), located 300 km east of Halifax, Nova Scotia in Canada (43°55'49''N, 60°00'67''W) (Figure 4.1). The island supports the world's largest breeding colony of grey seals (~370,000 individuals and an estimated 82,000 pups born annually) (Bowen et al. 2007; Hammill et al. 2017). The Sable Island population of grey seals has been the focus of long-term research dating back to the 1960s, with individual-based records obtained for a subset of the population. As weaned pups, individuals were selected randomly from the colony and permanently marked with a unique-character hot-iron brand applied to the lower back. Study animals are therefore of known age. The branding program occurs for two to three consecutive years about every ten years. Beginning annually in 1983, the entire island has been systematically searched for branded individuals. Each census is performed weekly from early December to late January, a period when females haul out to give birth and to mate. Subjects of our study were adult females born in the years 1974, 1985-1987, 1989, and 1998-2002 (Table C.1). Tissue samples were collected from a hind-flipper at the time of branding (i.e. as pups) for genetic analysis. For 33 females, tissue samples collected from the individual as a pup were lost, and thus required re-sampling as adults using a biopsy pole to obtain a skin scraping from the female's shoulder or hip area. All capture and handling techniques were in compliance with the Canadian Council on Animal Care and approved by Canada's Department of Fisheries and Oceans (DFO) and the University of Alberta's Animal Care Committees. Research was performed under permits issued by DFO Canada.

4.3.2 Boldness measurements

Boldness data were collected in consecutive breeding seasons during 2008-2018. Here, boldness was determined according to a female's response to a potentially risky situation, specifically in defense of her offspring (see Bubac et al. 2018). Females were assigned a boldness score from one to six following the advancement of three researchers toward the focal female and handling of her pup. A score of one represented shy individuals and of six very bold individuals (Table 4.1). Interactions lasted approximately one minute, giving researchers enough time to sex the pup and apply a uniquely numbered tag to the webbing of the pup's hind-flipper

for later identification. Each female was tested on day-three postpartum or later to permit adequate bonding between mother and offspring. Boldness scores were determined for 525 females over the 11-year period. Repeatability of boldness was estimated for this dataset with the R package 'MCMCglmm' (Hadfield 2010) (see Bubac et al. 2018 for details). With evidence that boldness is highly repeatable in this population (R = 0.581 [CI: 0.543-0.624]; N = 460 females with two or more boldness scores), and for the purposes of the current study, we selected a subset of females with the criteria of having extreme shy-bold phenotypes along the normal distribution of boldness values, as determined by a female's scores averaged across years (Table 4.1). Extremes of the shy-bold continuum were phenotypes falling in the shyest (average boldness scores of ≤ 2.5) and boldest (average boldness scores of ≥ 4) ranges, yielding 188 total individuals for which genetic material was available. To confirm this subset was appropriate, we performed analyses on residuals from a GLM correcting for sources of variation on boldness using the entire dataset of females (see Figure C.1). Upon selection of the shyest and boldest females, we considered repeated measures collected from the 2008-2018 sampling period.

We additionally recorded a female's birth-site habitat selection, characterized by the microhabitat type where a female and her pup resided. Sable Island has various microhabitat features and the density of individuals is not uniformly distributed over the island, wherein sandy shoreline and inland areas are more densely populated than vegetated dunes. We recorded birth-site habitat selection to account for differences that microhabitat feature, here a proxy of density, might have on boldness scores (Table C.2).

4.3.3 Maternal performance traits

For the 188 females behaviourally assayed, records of maternal performance traits (PWM and lactation duration) were available for up to 28 years (1991-2018). After locating branded females in the breeding colony during the weekly island-wide censuses as described above, study females were monitored daily to assess her reproductive status. A female's parturition date could be estimated from her pup's developmental stage, such that newborn pups (i.e. 'stage one' lasting approximately 1-2 days after birth) are characterized by having loose skin folds and yellowish pelage that is still wet from birth fluids (Bowen et al. 2003). We could not determine the parturition date for females first located with pups beyond stage one. Female-pup pairs were monitored daily, and from a distance following the behavioural assay/tagging of the pup to

reduce disturbance to the pair. Weaning and cessation of parental care is abrupt in the grey seal, with females abandoning her pup to return to the ocean. Daily monitoring, therefore, permitted identification of the weaning date, giving an estimate of lactation duration for females in which parturition date was known. Upon weaning, researchers identified the pup by its hind-flipper tag to confirm a focal female's pup. At this time, the pup was weighed to the nearest 0.5 kg and its sex confirmed. Phenotypic values for each trait (boldness, PWM, and lactation duration) were plotted and visually examined to ensure normality.

4.3.4 Candidate gene sequencing

Total genomic DNA was extracted from tissue using Qiagen DNeasy Blood and Tissue Kits according to the manufacturer's protocol (Qiagen, Valencia, California, USA). As no sequence information for the candidate genes of interest existed for grey seals, we designed several *de novo* forward/reverse primer pairs spanning each gene (i.e. *DRD4*, *SERT*, *OXTR*, *MC1R*, and *MC5R*) using homologous sequences from a closely related species, the Weddell seal (*Leptonychotes weddellii*) (*DRD4*: GenBank: XM_006741797.1; *SERT*: GenBank: XM_006734532.1; *OXTR*: GenBank: XM_006736571.1; *MC1R*: GenBank: XM_006746161.1; and, *MC5R*: GenBank: XM_006744833.1) in Geneious v. 11.1.5 (http://www.geneious.com, Kearse et al. 2012) (Supplemental Table S3). These primer sets were used to sequence both intron and exon gene structures of each candidate gene in grey seals (Figure 4.2).

To test the efficacy of primers and search for putative polymorphisms, we initially sequenced 24 individuals (12 very bold and 12 very shy females) for each gene. Fragments of each candidate gene were amplified using touchdown polymerase chain reactions (PCRs) in 20 µl reactions consisting of 10-100 ng genomic DNA, 10X PCR buffer, 25 mM $MgCl_2$, 0.2 mM forward and reverse primers, 2 mM dNTPs, and 0.05 U TopTaq DNA polymerase (Qiagen). Thermocycling parameters consisted of denaturation at 94°C for 4 min, followed by 24 cycles of 94°C x 1 min, 60°C x 30 s (successively decreased by 0.5°C with each cycle), 72°C x 2 min, then 15 cycles of 94°C x 1 min, 48°C x 30 s, 72°C x 2 min, and a final extension of 72°C x 10 min and 4°C soak. PCR products were visualized on a 1% agarose gel after ethidium bromide staining to ensure amplification of anticipated fragment size. Products were purified with Exonuclease and Shrimp Alkaline Phosphatase (ExoSap) enzymatic reactions (USB, Cambridge, Massachusetts, USA), and subsequently cycle-sequenced in both directions using amplification

primers and ABI Big Dye Terminator (Applied Biosystems, Inc., Foster City, California, USA). Cycle-sequencing conditions included the following: denaturation at 96°C for 3 min, and 45 cycles of 96°C for 10 s, 50°C for 05 s, and 60°C for 2 min. Reactions were purified using a standard ethanol precipitation to remove unincorporated dye terminators and electrophoresed on an ABI 3730 genetic analyzer (Applied Biosystems, Inc.). Sequences were aligned and visually examined for single nucleotide polymorphisms (SNPs) using Geneious. We used the National Center for Biotechnology Information's BLAST search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using the standard nucleotide BLAST (blastn) against the entire nucleotide database on all resulting candidate gene sequences to confirm gene identity, and align SNPs within coding/non-coding regions.

4.3.5 Genotyping

PCR and ExoSap reactions were performed for all 188 females according to methods described above. Following gel electrophoresis and PCR purification, we used SNaPshot reactions to genotype each individual at every known SNP location as identified from the initial panel of 24 females (Applied Biosystems, Inc.). SNP primers were created using Geneious, such that primers were immediately adjacent to the identified SNP, and were designed to contain a repeating T tail of varying lengths to ensure accurate differentiation and genotyping at each locus. PCR products were pooled to include groups of sequences that had compatible SNP primers (i.e. primers with differing lengths of T tails). SNaPshot reactions consisted of 4 µl pooled PCR products, 1 µl pooled forward primers (diluted to 2 uM), and 5 µl SNaPshot Multiplex Ready Reaction Mix (Applied Biosystems, Inc.). Reactions were amplified with the following protocol: 96°C for 3 minutes, and 25 cycles of 96°C x 10 s, 50°C x 5 s, and 60°C x 30 s. We performed ExoSap reactions to remove unincorporated ddNTPs. Finally, 3 µl of reaction products were combined with 8 µl of Hi-Di Formamide/Liz-120 bp size standard mix, denatured at 95°C for 2 minutes and electrophoresed on an ABI 3730 genetic analyzer (Applied Biosystems, Inc.). Genotypes for each individual were scored using GeneMapper 4.0 (Applied Biosystems, Inc.).

4.3.6 Genotype results

We used the online version of Genepop 4.7 (Raymond and Rousset 1995; Rousset 2008) to check each locus for deviations from Hardy-Weinberg equilibrium (HWE) and to test for pairwise linkage disequilibrium between variants. For each test, we raised the Markov chain default parameters to 10,000 dememorizations, 1000 batches, and 10,000 iterations. To account for multiple testing, significance levels were adjusted for false discovery rate (FDR) using the Benjamini-Hochberg method (Benjamini and Hochberg 1995) with the p.adjust function in R v3.6.1 (R Core Team 2019). As population stratification from genetic structure and relatedness may cause false positives in association tests (Laird and Lange 2011), we considered both population structure and relatedness. We completed a principal component analysis (see Figure C.2) to confirm our dataset was unstructured; however, we recognize our limited number (seven) of unlinked SNPs do not have sufficient marker resolution to estimate stratification. Previous studies on the genetics of Northwest Atlantic grey seals, including seals from our study population, found no evidence of population structure using microsatellites (Wood et al. 2011), mtDNA sequence data (Cammen et al. 2018a), and a large SNP panel (8700 loci; Cammen et al. 2018b). The nature of the longitudinal dataset (individual-based records for branded females) highlighted six mom-daughter pairs; therefore, mom or daughter were removed from statistical analyses. The female with less missing genotypic data across the five genes was retained for analysis.

4.3.7 Genetic diversity and neutrality tests

Genetic diversity indices and Tajima's *D* neutrality tests (Tajima 1989) were estimated using DnaSP v6.12 (Rozas et al. 2017) for the sequence data. Positive Tajima's *D* values are indicative of balancing selection and/or a decrease in the overall size of a population whereas negative values infer positive selection and/or population expansion. For each SNP locus, we calculated expected and observed heterozygosities using Genepop.

4.3.8 Association tests

Generalized linear mixed-effects models (GLMMs) were used to test for the effects of genotypes on boldness, PWM, and lactation duration. Models were run using the package 'lme4' (Bates et al. 2015) in R, with ML and Satterthwaite's t-test method implemented in the 'afex'

package (Singmann et al. 2019). We used the additive model (each additional copy of the variant allele increases the response), and the overdominance model (genotype categories coded as heterozygotes versus homozygotes). For all models, genotypes were fitted as fixed factors in separate, gene-specific models using only loci that passed quality control filters (i.e. unlinked and polymorphic loci).

Boldness was scored on an ordinal scale, but effectively represents an axis of continuous values along a shy-bold continuum of behavioural variation. Therefore, boldness was fitted as a continuous response variable. We included year and female identity as random factors to account for interannual variation and repeated measures on the same individual taken through time, respectively. In addition to SNP genotype(s), we fitted maternal age as a fixed factor, as it has been shown that older grey seal females are on average bolder than younger individuals (Bubac et al. 2018). Age was grouped into biologically relevant, five-year interval bins, with the oldest females aged ≥ 26 years pooled into one group. This resulted in a 5-level factor: bin 1 = ≤ 10 years (females are young and less experienced); bin 2 = 11-15 years (females are young with more experience); bin 3 = 16-20 years (females in prime reproductive years); bin 4 = 21-25 years (females in prime reproductive years); bin 5 = ≥ 26 years (females are old with some evidence of senescence) (Bowen et al. 2006). We also fitted birth-site habitat selection as a 3-level fixed factor (Table C.2), given that microhabitat features account for boldness variation in the Sable Island population (Bubac et al. 2018).

It has been found that bold females generally wean heavier pups (Bubac et al. 2018), a relationship supported with our reduced dataset ($t = 2.999$; $P = 0.004$, $r = 0.10$; $n = 176$ females). Additionally, a relationship exists between boldness and lactation duration ($t = 2.559$; $P = 0.011$, $r = 0.18$; $n = 118$ females) (Figures C.3 and C.4), substantiating the investigation of gene-PWM/lactation duration associations to explore whether the same underlying mechanisms are at play. In models assessing the genetic effects on life-history traits, PWM and lactation duration were fitted as continuous response variables in individual models, with year and identity included as random factors. As maternal age is expected to affect reproductive performance (Bowen et al. 2006), we fitted age again as a 5-level fixed factor (see above). In the model including PWM as the response variable, we further fitted pup sex as a 2-level fixed factor to account for previous findings that male pups are often heavier than female pups at weaning (Bowen et al. 2006; Bubac et al. 2018).

Importance of variables was assessed based on both estimates of significance and whether the SNP confidence interval included zero. Model selection was performed using Akaike's information criterion values (Table C.4). We further estimated effect size (r) for each SNP using the equation r = sqrt(t^2/(t^2 + df)); however, these values should be interpreted with caution when using GLMMs (Jaeger et al. 2016). The biological significance of effect sizes were evaluated following suggestions by Cohen (1988), wherein a small effect is represented by r = 0.1, a medium effect by r = 0.3, and a large effect by r = 0.5 (Møller and Jennions 2002). Lastly, to estimate the proportion of trait variation explained by each gene and the other fixed factors, as well as all loci combined, we calculated marginal R^2 ($R^2_{GLMM(m)}$) according to Nakagawa and Schielzeth (2013).

4.4 Results

We obtained quality genotypic data for 185 grey seal females; however, removing individuals to account for relatedness left 180 females for association analyses. The age of females with corresponding behavioural and maternal performance records ranged from 4 to 37 years, with an average age of 14 years (Table C.1). A total of 1183 behavioural scores along the shy-bold continuum were recorded for this subset of females over an 11-year study period (Table 4.1). Measures of PWM and lactation duration were available from 1991-2018 (28 years) for 177 females (1496 observations) and 152 females (544 observations), respectively. Two records of lactation duration from two females were exceptionally low (1 and 4 days), resulting from storms in 2002 and 2012 that caused premature female-pup separation. As such, these outliers were removed from analyses, leaving 542 observations from 151 females.

From the 24 females initially sequenced (GenBank accession numbers MW864572-MW864597), we detected a total of 36 SNPs. We sequenced the majority of the *DRD4* gene (86% of gene sequenced) and detected three SNP variants in intron 3 (Figure 4.2). A total of 26 SNPs (23 intronic and 3 exonic) were identified in *SERT* (45% sequenced), three intronic SNPs in *OXTR* (13% sequenced), three exonic SNPs in *MC1R* (61% sequenced), and one exonic SNP in the *MC5R* gene (47% sequenced). Overall, the sequenced gene regions had low nucleotide diversity (π = 0 to 0.00628, with an overall mean of π = 0.001) (Table C.5). In the analysis of Tajima's *D*, we found eight regions (*MC5R*, one segment in *DRD4*, two in *OXTR*, and four in

SERT) to be under positive selection according to the sign of Tajima's *D*. The other ten regions were indicative of balancing selection (*MC1R*, one in *DRD4*, and eight in *SERT*), wherein one sequenced region of *SERT* was statistically significant ($P < 0.05$) (Figure 4.2; Table C.5).

Out of the total 36 SNPs detected and assayed between the five genes in all 185 individuals, three variants from *DRD4*, 13 from *SERT*, and one variant from *MC1R* were retained for analysis (Table 4.2), as the other loci were effectively monomorphic in our study subset [10 SNPs; minor allele frequency threshold < 0.01], or failed to amplify using the SNaPshot protocol (9 SNPs). This failure rate is comparable to those reported in other studies using SNaPshot (Pati et al. 2004). Five loci deviated significantly from HWE ($P < 0.05$) (*MC1R*_126, *SERT*_11432, *SERT*_15636, *SERT*_7540, and *SERT*_11689), which reduced to four following correction for multiple testing (Table 4.2), indicating that some genotypes were absent or under-represented. This may be due to sequencing errors; yet, these SNPs are potentially associated with the fitness-related traits, and therefore may be under selection and expected to deviate from HWE. An extreme excess proportion of heterozygotes at one of the deviating *SERT* loci, *SERT*_11689, was suggestive of a paralog, so was excluded from further analysis. We retained the remaining HWE deviating SNPs for analysis.

Following correction for multiple testing, 35 locus-pairs were significantly linked ($P < 0.05$), and all linked loci were within *SERT* (Table C.6), potentially indicating a hitchhiking effect. Among the *SERT* SNPs, we observed two that were not linked while the remainder represented one linkage group and therefore we retained one SNP from this group. This yielded a total of seven unlinked-SNPs between three genes (*DRD4*, *SERT*, and *MC1R*) for association analyses. Removal of linked loci in turn removed two of the loci out of HWE (*SERT*_11432 and *SERT*_7540). Genotype and allele frequencies for each SNP retained are presented in Table 4.2.

4.4.1 Association tests

We found a significant additive allele effect ($P < 0.05$), and a second locus that showed a marginally non-significant association ($P < 0.1$) between boldness and SNPs in the *SERT* gene (S*ERT*_12472 and *SERT*_15636, respectively) (Table 4.3; Table C.7). At locus *SERT*_12472 (intron two), shy female seals lacked the major allele (G). In the suggestive link between *SERT*_15636 (exon four) and boldness (Figure 4.3; Figure C.5), the homozygote minor allele genotype (AA) was associated with the bold phenotype. The estimated effect size of

*SERT*_12472 was 0.189 and of *SERT*_15636 was 0.137, suggesting moderate genetic effects. Variance in boldness accounted for by *MC1R* ($R^2_{GLMM(m)} = 9.48 \times 10^{-5}$) was low, whereas *DRD4* ($R^2_{GLMM(m)} = 0.0199$) and *SERT* ($R^2_{GLMM(m)} = 0.0345$) explained more. Altogether, genotypes of the three genes explained 13.66% of boldness variation. Age and habitat further explained 2.85% and 0.57% variance in boldness, respectively (Table C.8).

For maternal performance, we found one SNP within *DRD4* (*DRD4*_1363 in intron three) that was marginally non-significantly associated with PWM (P = 0.051; r = 0.232) (Table 4.3), with homozygote minor allele (TT) individuals weaning pups on average 3.2 kg more than homozygote major allele (CC) conspecifics (Figure 4.4). While not significant, trends existed at locus *SERT*_12472 and *SERT*_15636, such that females with the minor allele (A) weaned lighter pups than GG females (Figure C.6). Overall, 6.52% of total variation in PWM was explained by genetic effects of the seven loci. *MC1R* and *SERT* explained very little PWM variance ($R^2_{GLMM(m)} = 0.00125$ and 0.00648, respectively), while *DRD4* ($R^2_{GLMM(m)} = 0.0178$) had a more appreciable genetic effect. *SERT*_12472 also showed a trend with lactation duration (r = 0.127) (Table 4.3; Figure 4.4), with individuals having the minor allele (A) exhibiting shorter lactation durations (Figure C.7). Much less total variance in lactation duration was explained by *MC1R* ($R^2_{GLMM(m)} = 0.00014$), *DRD4* ($R^2_{GLMM(m)} = 0.00977$), and *SERT* ($R^2_{GLMM(m)} = 0.00677$) (overall gene effect: $R^2_{GLMM(m)} = 0.106$) (Table C.8).

We found three marginally non-significant associations based on overdominance models for all three phenotypic traits examined (Tables C.9-C.11). *SERT*_12472 had moderate overdominant genetic effects on boldness (r = 0.148) and lactation duration (r = 0.188), and *DRD4*_1853 had a moderate effect on PWM (r = 0.204). To ensure that other variants within the *SERT* linkage group did not have a greater effect or higher level of association with the traits examined, we re-ran models including alternate SNPs linked with locus *SERT*_5987. These alternate loci demonstrated similar effect sizes to those presented for association between *SERT*_5987 and boldness, PWM, and lactation duration (Table C.12). Lastly, to validate that rare genotypes were not driving the relationships observed, we re-fitted rare genotypes at the *DRD4*_1363, *SERT*_12472, and *SERT*_15636 loci, grouping the minor allele homozygote genotype with the heterozygote genotype and also eliminating the rare genotype from analyses altogether (Figures C.8-C.10).

4.5 Discussion

Exploring the genetic basis of quantitative traits in free-ranging populations is challenging given the simultaneous influence of environmental variables and state-dependent factors (e.g., size, body condition, and energy reserves). Nevertheless, advances in molecular genetics and analytical techniques can provide insights on genotype-phenotype links. Here, we used long-term boldness and life-history data collected on grey seals to examine relationships between phenotype and genetic variation of five candidate genes in an effort to shed light on the mechanisms behind the coexistence of different phenotypes within a free-ranging pinniped population. We used genomic resources from a closely related species, the Weddell seal, to detect SNPs in grey seal orthologous sequences of the following candidate genes: *DRD4, SERT, OXTR*, *MC1R,* and *MC5R*. Association analyses revealed a relationship between *SERT* and shy-bold phenotypes. Furthermore, we found evidence that *DRD4* and *SERT* likely play a role in maternal performance traits. To the best of our knowledge, our association study is the first to explore the association between genetics and behavioural variation and maternal performance characteristics in a wild marine mammal population, extending the candidate gene literature to include more taxonomic diversity.

4.5.1 Genotype-phenotype associations

We found a relationship between *SERT* and risk-taking behaviour in female grey seals, adding to a growing list of studies documenting the general role and conserved function that *SERT* has on the expression of behavioural traits (e.g., great tits; Riyahi et al. 2015 and Timm et al. 2018). Boldness was linked to a SNP genotype at *SERT*_12472 in intron two, such that individuals with the minor allele (A) were generally shyer than major allele homozygotes (GG). Polymorphisms in intron two of *SERT* have been linked with changes in transcription and alternative splicing, as well as the regulation and efficiency of gene expression, affecting various behaviours in other mammalian species, including humans (MacKenzie and Quinn 1999; Battersby et al. 1996). Holtmann et al. (2016) similarly discovered a number of significant associations between loci in intronic regions and boldness in their study on a wild bird population, and highlight the importance of targeting various gene regions for candidate gene studies.

The test we used to assess boldness stimulated a natural response of offspring protection in seals assayed, effectively measuring a component of parental care. Therefore, the genotype-boldness relationship discovered may provide insight into the behavioural functional link between personality and other behaviours, including parental care (Réale 2007). In support of this, associations between boldness and weaning mass and lactation duration have been established in the grey seal, with bolder females generally exhibiting longer lactation durations and weaning heavier pups than more timid individuals (Bubac et al. 2018). The biological significance of this is nontrivial. Grey seal pup survival is dependent upon acquiring sufficient mass (i.e. blubber) from maternal milk energy transfer during the lactation period (Iverson et al. 1993; Mellish et al. 1999). Upon weaning, the percent fat gained supports the pup through a post-weaning fast that lasts until the individual is capable of foraging independently (on average ~3 weeks; Noren et al. 2008). As such, body condition and size at weaning increases the pup's probability of survival and recruitment to the population (Hall et al. 2001; Bowen et al. 2015). That a significant relationship exists between boldness, PWM, and lactation duration does not necessarily indicate that the same underlying molecular mechanisms are at play. For instance, while female grey seals exhibit consistent individual differences in behaviour, they also vary in their physiological capacity to deliver milk (Lang et al. 2009); thus, creating an impetus to examine specific genotype-phenotype relationships.

We discovered a suggestive link between variations in *SERT* (*SERT*_12472) and lactation duration, a finding consistent with other patterns that we detected at this locus wherein the rare type is generally associated with shyness and lower weaning mass. Serotonin affects affiliative responses towards offspring by its influence on an individual's disposition and behavioural decisions (Emiliano et al. 2007; Bakermans-Kranenburg and van Ijzendoorn 2008), and other studies have similarly reported a correlative relationship between serotonin genes and fitness-related traits in wild vertebrate populations (e.g., see Prasad et al. 2015; Timm et al. 2018). We further detected a correlation between *DRD4* genotypes and PWM. The dopaminergic system has been a system commonly studied for the role it plays in behavioural, cognitive, and locomotive variation; yet, the definitive function of *DRD4* remains uncertain and its biological significance varied in different systems and environments (Oak et al. 2000; Korsten et al. 2010; Riyahi et al. 2017). While the fitness-related traits examined in this study may be partly modulated by behavioural decisions via *SERT* and *DRD4,* it is also possible that results observed

may be due to the influence of the serotoninergic and dopaminergic systems on the regulation of other hormones (Emiliano et al. 2007).

The serotonergic system, for example, mediates the release of oxytocin (Jorgensen et al. 2003), a neuropeptide hormone that plays a crucial role in promoting parturition and lactation as well as developing mother-offspring social bonds (Kendrick 2000; Lim and Young 2006). Natural and experimental studies performed on another population of grey seals have established a link between levels of oxytocin in females and the likelihood of pup separation, aggressive acts towards conspecifics, and strength of maternal bonds with offspring (Robinson et al. 2015; Robinson et al. 2017); thus, making *OXTR* an interesting gene with which to explore its genetic effect on maternal phenotypic variation. Unfortunately, we only sequenced ~13% of *OXTR* given difficulties encountered designing primers from Weddell seal genomic resources and non-specific amplification, preventing complete interrogation of the *OXTR* gene and detection of genetic variants with which to perform association analyses. Nevertheless, the continued and rapid development of genomic resources, as recently reported among marine mammals (Cammen et al. 2016), show promising potential to provide a complete and annotated reference genome for the grey seal, or closely related species, that will permit further unravelling the relationship between *SERT, OXTR*, and maternal performance traits.

Which behavioural traits are favored is likely a product of specific spatial, temporal, and contextual influences (Wolf and Weissing 2010), such that, for example, shy or bold behaviours may be selected against in one context or situation but favorable in other instances (e.g. foraging, reproduction, anthropogenic conflict) (Sih et al. 2004; Wolf et al. 2007). It is therefore likely that fluctuating selection pressures dependent upon particular contexts is driving the behavioural variation that we observed. Tajima's *D* values and excess sequence variation (notably at *SERT*) support the occurrence of balancing selection, and thus, have consequences for the underlying genetic composition of this population. Prior studies have reported similar patterns of selection on behaviour-related genes, including those underlying dopaminergic and serotonergic function (Howell et al. 2007; Chakraborty et al. 2010). In blackbirds, (*Turdus merula)*, for instance, harm avoidance traits found to be associated with a *SERT* polymorphism were subject to selection pressures in a novel environment, where rare alleles had a selective advantage in a population undergoing an urbanization event (Mueller et al. 2013). However, our results indicating balancing selection should be interpreted with caution. Demographic processes, including

bottleneck events as experienced by Northwest Atlantic grey seals (Cammen et al. 2018b), can yield similar neutrality test values (Maruyama and Fuerst 1985). Given our preliminary results, it will be interesting to track changes in the genetic composition of the Sable Island population over time, as well as to test for these genetic effects in other populations of grey seals.

4.5.2 Limitations

Like many marine mammals, Northwest Atlantic grey seals have undergone a severe bottleneck with its genetic signature evident today (Cammen et al. 2018b). We discovered SNPs that were infrequent in sequences of females examined as to be effectively uninformative in association analyses, which is consistent with the expectation of many rare genetic variants from rapid growth and expansion during recovery after a bottleneck event (Nei and Li 1976; Maruyama and Fuerst 1985). Furthermore, molecular diversity indices as estimated across the sequenced regions revealed low variation, a result not unexpected given the demographic history of the grey seal. The nature of the genetic structures (i.e. coding versus non-coding) sequenced may contribute to low diversity values. Though *SERT* had an appreciable amount of intronic regions sequenced, much of the other sequenced regions of the remaining genes were primarily exonic where less variation is typically detected (Figure 4.2). As such, *SERT* was more diverse than the other genes, possibly explained by different selection pressures between exons and introns. Still yet, without an annotated reference genome for the grey seal, we relied on primers designed from a closely related species that diverged approximately 15 million years ago (Fulton and Strobeck 2010), likely reducing primer specificity and gene coverage. This, combined with long repetitive regions in certain genes sequenced, contributed to difficulty in obtaining high-quality sequencing data, an issue frequently encountered among non-model organisms (Garvin et al. 2010; Helyar et al. 2011).

While it is possible that we may not have had enough power to resolve certain gene-phenotype relationships, other candidate gene studies have also been met with heterogeneous results, demonstrating the complex relationship between genes and quantitative traits (e.g. Edwards et al. 2015; Korsten et al. 2010). Many quantitative traits are polygenic, wherein detection of significant associations may be biased towards loci of larger effect size (Göring et al. 2001; Wellenreuther and Hansson 2016). The amount of variation in boldness, PWM, and lactation duration accounted for by *SERT* and *DRD4* likely explains an important fraction of the

moderate-to-high repeatability measures of these traits (R = 0.48-0.61) (Lang et al. 2009; Bubac et al. 2018), and of what heritability might be, in the Sable Island population of grey seals. Yet, with the expectation that personality traits are controlled by many genes of small effect (Laine and van Oers 2017), other genes [e.g., arginine vasopressin receptor 1A (*AVPR1A*) and monoamine oxidase A (*MAO-A*)] not explored herein could be contributing to the behavioural variance observed, and the unaccounted variability in the observed grey seal phenotypes deserves further attention. Although candidate genes offer important preliminary information, a genome-wide association analysis should be done in an effort to further resolve the molecular genetic basis of these fitness-related traits.

4.5.3 Conclusion

Despite the difficulties, it is important to study the genetic basis of complex traits across a variety of species in an effort to determine whether particular molecular mechanisms underlying these traits are evolutionarily conserved (Bengston et al 2018). Only by examining the genetic basis of boldness and other fitness-related traits across taxonomically diverse species will we be able to discern whether such mechanisms are common to all taxa (Fidler 2011), or are unique to specific populations of a particular species. This research may also provide insight into the processes shaping and maintaining individual phenotypic variation in wild populations, thereby allowing researchers to assess the capacity of populations to adapt in response to changing environmental conditions and other selection pressures. Individual-based data records and archived tissue samples from a longitudinal study enabled us to test for a link between fitness-related traits and genotype in a species of marine mammal. This underscores the importance of pre-existing long-term studies for unravelling the molecular genetic basis of quantitative traits in free-ranging species.

Table 4.1: Boldness scores with descriptions of behaviour, and the associated number of observations collected for each score as well as the number of Sable Island grey seal females. Of the 180 females with repeated measures obtained from 2008-2018, the average score for shy individuals was 1.789 (SE ± 0.187; n = 95 females with 478 observations), while that of bold individuals was 4.782 (SE ± 0.0332; n = 85 females with 527 observations).

Score	Behaviour	Observations
1	Shy; flees, quickly moves > 2 m away from researchers and pup	101
2	Shy; moves away < 2 m away from researchers and pup	377
3	Intermediate; stays nearby and shows no boldness	178
4	Mild boldness; vocalizes and makes abrupt movements towards researchers	223
5	Moderate boldness; vocalizes, lunges towards researchers, displays open mouth threat	196
6	Extreme boldness; vocalizes, displays an open mouth threat, lunges and attempts to bite	108

Table 4.2: Allele and genotype frequencies for both linked and unlinked loci detected between three candidate genes (*DRD4*, *SERT*, and *MC1R*) in 180 female grey seals of Sable Island, Nova Scotia (Canada). Only unlinked loci were included in association analyses.

Locus	Major/minor allele	Location	MAF	H_{Obs}	H_{Exp}	HW P-value	Genotype (frequency)			Protein coding
DRD4										
*DRD4*_1363[a]	C/T	Intron 3	0.116	0.17	0.21	0.168	CC (0.8)	CT (0.17)	TT (0.032)	-
*DRD4*_1496[a]	G/A	Intron 3	0.389	0.48	0.48	1.000	GG (0.37)	AG (0.47)	AA (0.15)	-
*DRD4*_1853[a]	C/T	Intron 3	0.331	0.37	0.44	0.204	CC (0.48)	CT (0.37)	TT (0.14)	-
SERT										
*SERT*_2946	G/C	Intron 1	0.364	0.41	0.46	0.246	GG (0.43)	GC (0.41)	CC (0.16)	-
*SERT*_5987[a]	T/C	Intron 1	0.393	0.42	0.48	0.246	TT (0.4)	TC (0.42)	CC (0.18)	-
*SERT*_6056	G/T	Intron 1	0.356	0.44	0.46	0.700	GG (0.42)	GT (0.44)	TT (0.14)	-
*SERT*_6147	A/G	Intron 1	0.396	0.46	0.48	0.700	AA (0.38)	AG (0.46)	GG (0.17)	-
*SERT*_7235	A/T	Intron 1	0.261	-	-	-	AA (0.00)	AT (0.52)	TT (0.48)	-
*SERT*_7535	G/A	Intron 1	0.328	0.39	0.41	0.246	GG (0.48)	GA (0.39)	AA (0.13)	-
*SERT*_7540	A/G	Intron 1	0.210	0.19	0.33	0.000	GG (0.12)	GA (0.18)	AA (0.70)	-
*SERT*_11432	T/G	Intron 1	0.125	0.19	0.22	0.146	TT (0.78)	TG (0.18)	GG (0.03)	-
*SERT*_11673	C/G	Exon 2	0.138	0.25	0.24	0.700	CC (0.74)	CG (0.25)	GG (0.011)	Nonsyn
*SERT*_11689	C/T	Exon 2	0.478	0.92	0.50	0.000	CC (0.06)	CT (0.92)	TT (0.016)	Nonsyn
*SERT*_12472[a]	G/A	Intron 2	0.0819	0.13	0.15	0.204	GG (0.85)	AG (0.13)	AA (0.017)	-
*SERT*_15549	G/A	Intron 3	0.409	0.43	0.48	0.270	GG (0.38)	AG (0.43)	AA (0.19)	-
*SERT*_15636[a]	G/A	Intron 3	0.308	0.53	0.43	0.003	GG (0.43)	AG (0.53)	AA (0.04)	-
*SERT*_16657	C/G	Intron 4	0.286	0.41	0.41	1.000	CC (0.51)	CG (0.41)	GG (0.082)	
MC1R										
*MC1R*_126[a]	T/C	Exon 1	0.451	0.68	0.50	0.001	CC (0.11)	CT (0.68)	TT (0.21)	Syn
*MC1R*_740	A/T	Exon 1	0.348	-	-	-	AA (0.31)	AT (0.69)	TT (0.00)	Nonsyn

[a] Unlinked loci that were included in association analyses. Syn = synonymous; Nonsyn = nonsynonymous

Table 4.3: Additive model results for assessing the genetic association of three candidate genes (*DRD4, MC1R,* and *SERT*) with boldness, pup weaning mass (PWM), and lactation duration in female grey seals of Sable Island, Nova Scotia (Canada). Full model results with additional fixed factors (age, habitat, and pup sex) are provided in Supplemental Table S7. Female identity and year were included in models as random factors.

	Fixed factors	Estimate	SE	df	t	P-value	2.5% CI	97.5% CI	Effect size (r)
Boldness ~ SNP(s) + Age + Habitat; 180 females, 1183 total observations									
MC1R	**(Intercept)**	**2.978**	**0.288**	**117.929**	**10.345**	**< 0.0001**	**2.408**	**3.551**	**0.690**
	*MC1R*_126	-0.193	0.226	97.983	-0.856	0.394	-0.641	0.254	0.086
DRD4	**(Intercept)**	**3.040**	**0.422**	**88.629**	**7.204**	**< 0.0001**	**2.205**	**3.877**	**0.608**
	*DRD4*_1853	-0.197	0.202	75.629	-0.975	0.333	-0.599	0.203	0.111
	*DRD4*_1496	-0.073	0.255	77.416	-0.285	0.776	-0.579	0.435	0.0324
	*DRD4*_1363	0.255	0.307	74.517	0.830	0.409	-0.355	0.864	0.0957
SERT	**(Intercept)**	**1.979**	**0.558**	**156.060**	**3.544**	**< 0.0001**	**0.878**	**3.081**	**0.273**
	***SERT*_12472**	**0.626**	**0.267**	**149.329**	**2.346**	**0.020**	**0.100**	**1.152**	**0.189**
	*SERT*_5987	0.049	0.138	151.509	0.357	0.722	-0.222	0.320	0.029
	SERT_15636	*-0.303*	*0.180*	*149.401*	*-1.688*	*0.093*	*-0.657*	*0.051*	*0.137*
PWM ~ SNP(s) + Age + Sex; 177 females, 1496 total observations									
MC1R	**(Intercept)**	**47.503**	**1.480**	**117.636**	**32.089**	**< 0.0001**	**44.560**	**50.417**	**0.947**
	*MC1R*_126	0.381	1.062	90.016	0.359	0.721	-1.716	2.498	0.038
DRD4	**(Intercept)**	**49.416**	**1.578**	**86.415**	**31.307**	**< 0.0001**	**46.275**	**52.572**	**0.959**
	*DRD4*_1853	-0.726	0.730	65.650	-0.995	0.323	-2.178	0.726	0.122
	*DRD4*_1496	-0.984	0.924	68.000	-1.065	0.291	-2.828	0.857	0.128
	*DRD4*_1363	*2.274*	*1.146*	*69.383*	*1.985*	*0.051*	*0.00399*	*4.561*	*0.232*

SERT	**(Intercept)**	**45.347**	**2.533**	**144.528**	**17.902**	**< 0.0001**	**40.350**	**50.349**	**0.830**
	*SERT*_12472	0.177	1.196	133.524	0.148	0.883	-2.184	2.538	0.013
	*SERT*_5987	0.722	0.620	133.945	1.164	0.246	-0.502	1.945	0.100
	*SERT*_15636	1.002	0.802	131.035	1.249	0.214	-0.587	2.583	0.108
Lactation duration ~ SNP(s) + Age; 151 females, 542 total observations									
MC1R	**(Intercept)**	**15.629**	**0.408**	**65.749**	**38.293**	**< 0.0001**	**14.823**	**16.473**	**0.978**
	*MC1R*_126	-0.028	0.291	65.567	-0.097	0.923	-0.615	0.545	0.012
DRD4	**(Intercept)**	**16.498**	**0.493**	**67.510**	**33.476**	**< 0.0001**	**15.525**	**17.491**	**0.971**
	*DRD4*_1853	0.043	0.233	55.322	0.186	0.853	-0.420	0.510	0.025
	*DRD4*_1496	-0.267	0.308	70.740	-0.867	0.389	-0.878	0.346	0.103
	*DRD4*_1363	0.053	0.401	78.421	0.133	0.894	-0.744	0.850	0.015
SERT	**(Intercept)**	**14.688**	**0.701**	**133.107**	**20.956**	**< 0.0001**	**13.310**	**16.088**	**0.876**
	*SERT*_12472	0.469	0.337	118.074	1.391	0.167	-0.205	1.131	0.127
	*SERT*_5987	0.133	0.172	117.297	0.772	0.442	-0.207	0.477	0.071
	*SERT*_15636	0.015	0.224	116.217	0.069	0.945	-0.430	0.456	0.006

*Bolded values indicate statistical significance ($P < 0.05$). Italicized values are marginally non-significant ($P < 0.10$)

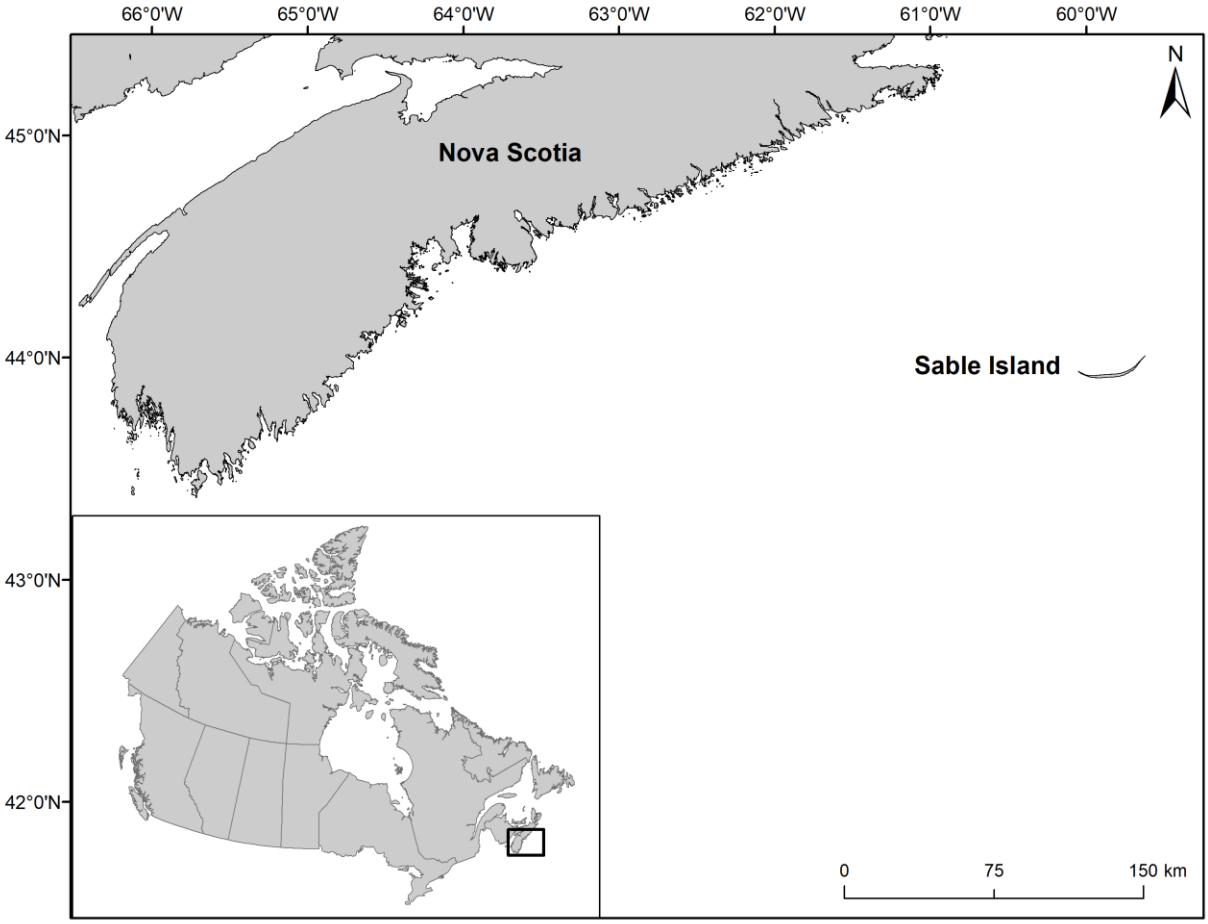

Figure 4.1: Map showing the location of Sable Island National Park Reserve of Nova Scotia, Canada where female grey seals have been monitored annually since the 1980s and behaviourally assayed from 2008-2018.

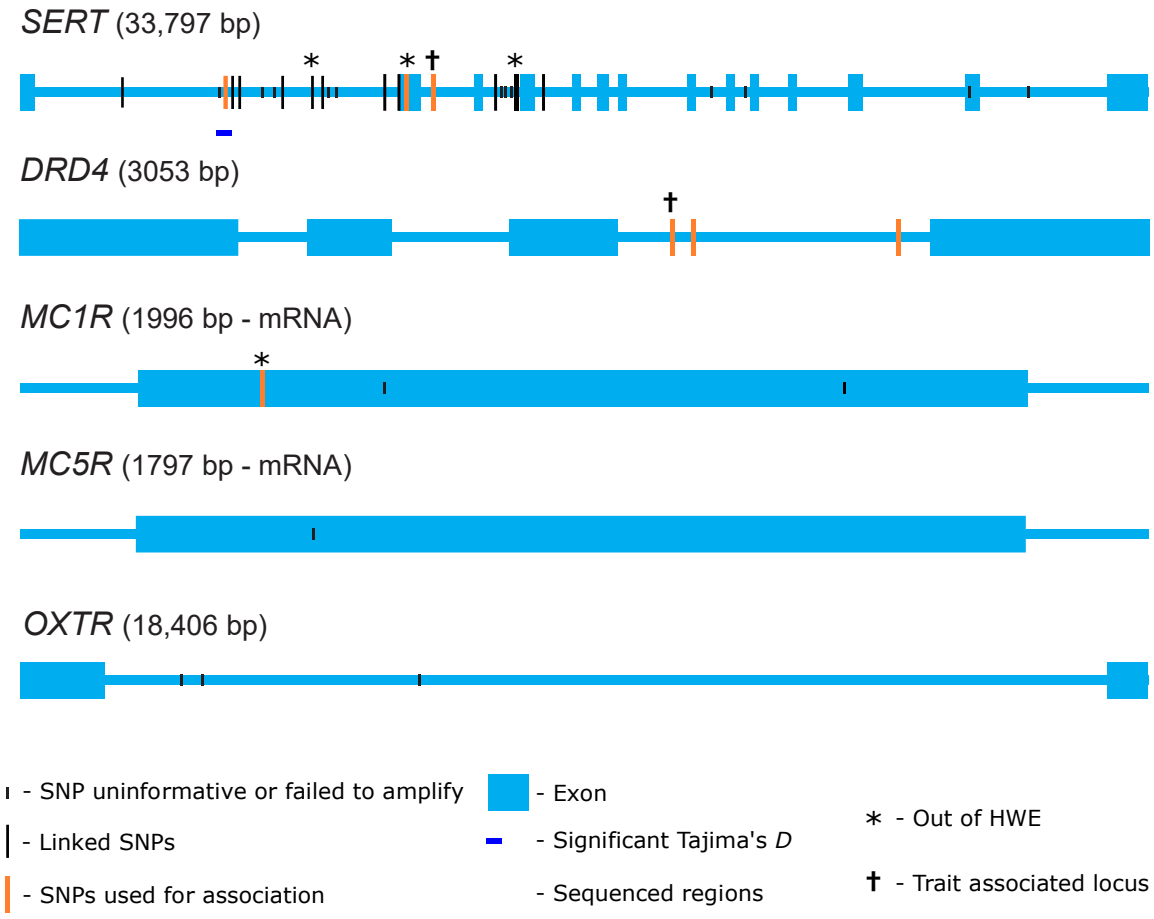

Figure 4.2: Schematics of gene structure for five candidate genes [serotonin transporter (*SERT*), dopamine receptor D4 (*DRD4*), melanocortin receptors 1 and 5 (*MC1R* and *MC5R*), and oxytocin receptor (*OXTR*)] used to assess the relationship between genotype and boldness and maternal performance variation in female grey seals of Sable Island, Nova Scotia (Canada).

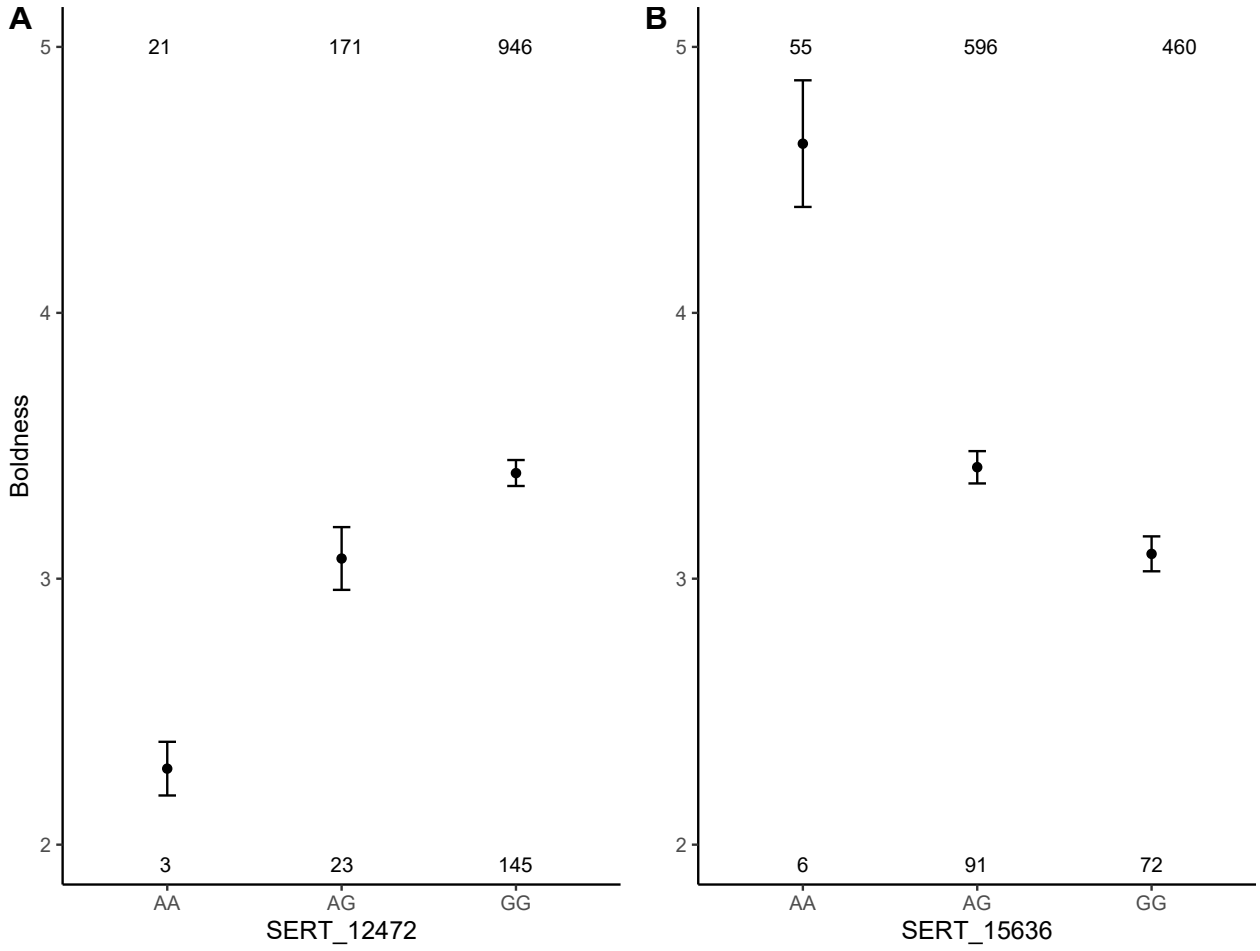

Figure 4.3: Boldness of female grey seals and the additive allele effect of the serotonin transporter (*SERT*) gene. Boldness showed associations with genotypes at: A) the *SERT*_15636 locus; and, B) the *SERT*_12472 locus. Sample size of females used for analyses are included above each genotype and number of behavioural observations are above the genotype mean. Error bars represent standard error of the genotype mean. The corresponding test statistics are provided in Table 4.3.

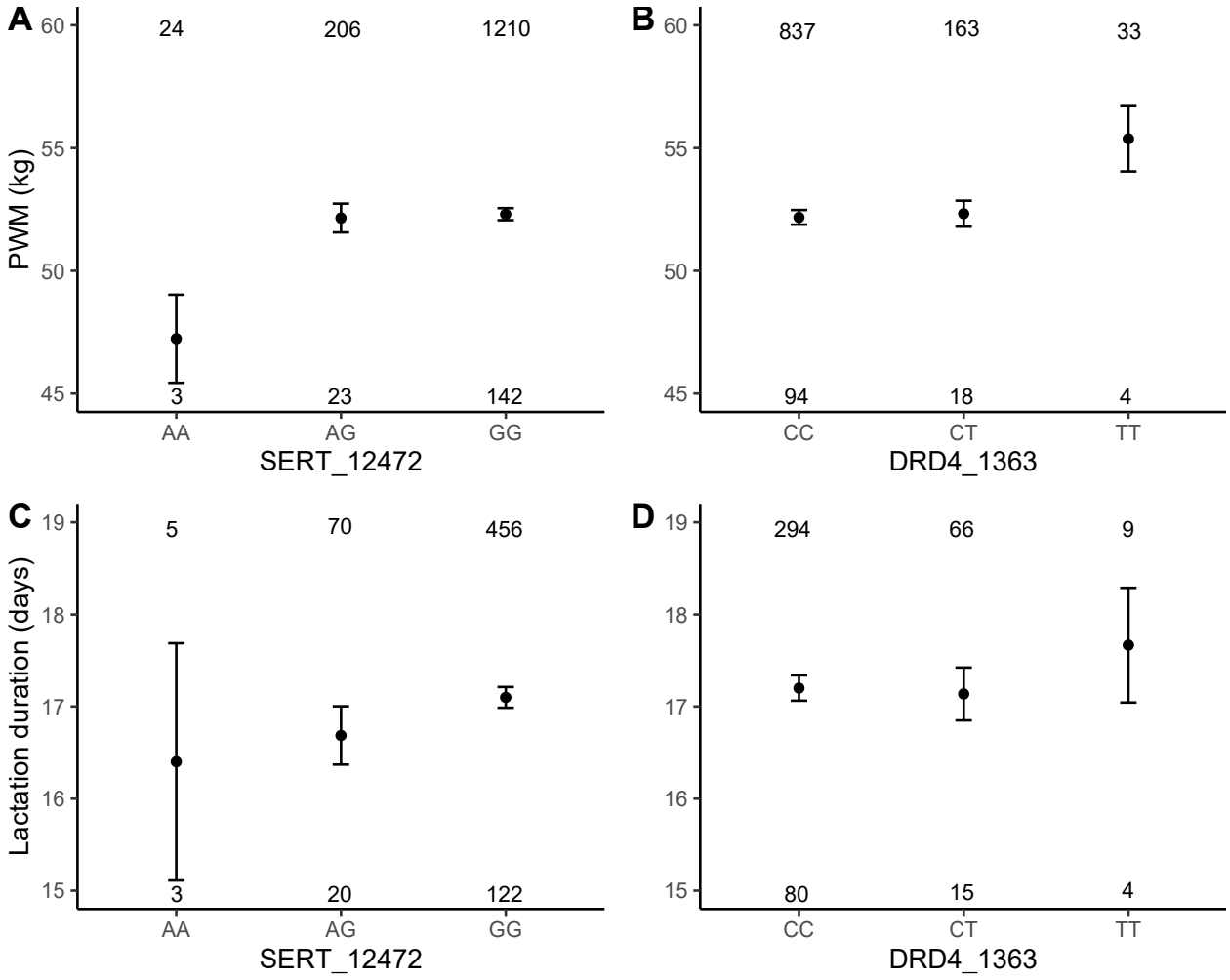

Figure 4.4: Relationship between the additive allele effect and two measures of grey seal maternal performance, pup weaning mass (PWM) (A and B) and lactation duration (C and D), at loci of two candidate genes that showed trends with each performance trait. Sample size of females used for analyses are included above each genotype and number of behavioural observations are above the genotype mean. Error bars represent standard error of the genotype mean. The corresponding test statistics are provided in Table 4.3.

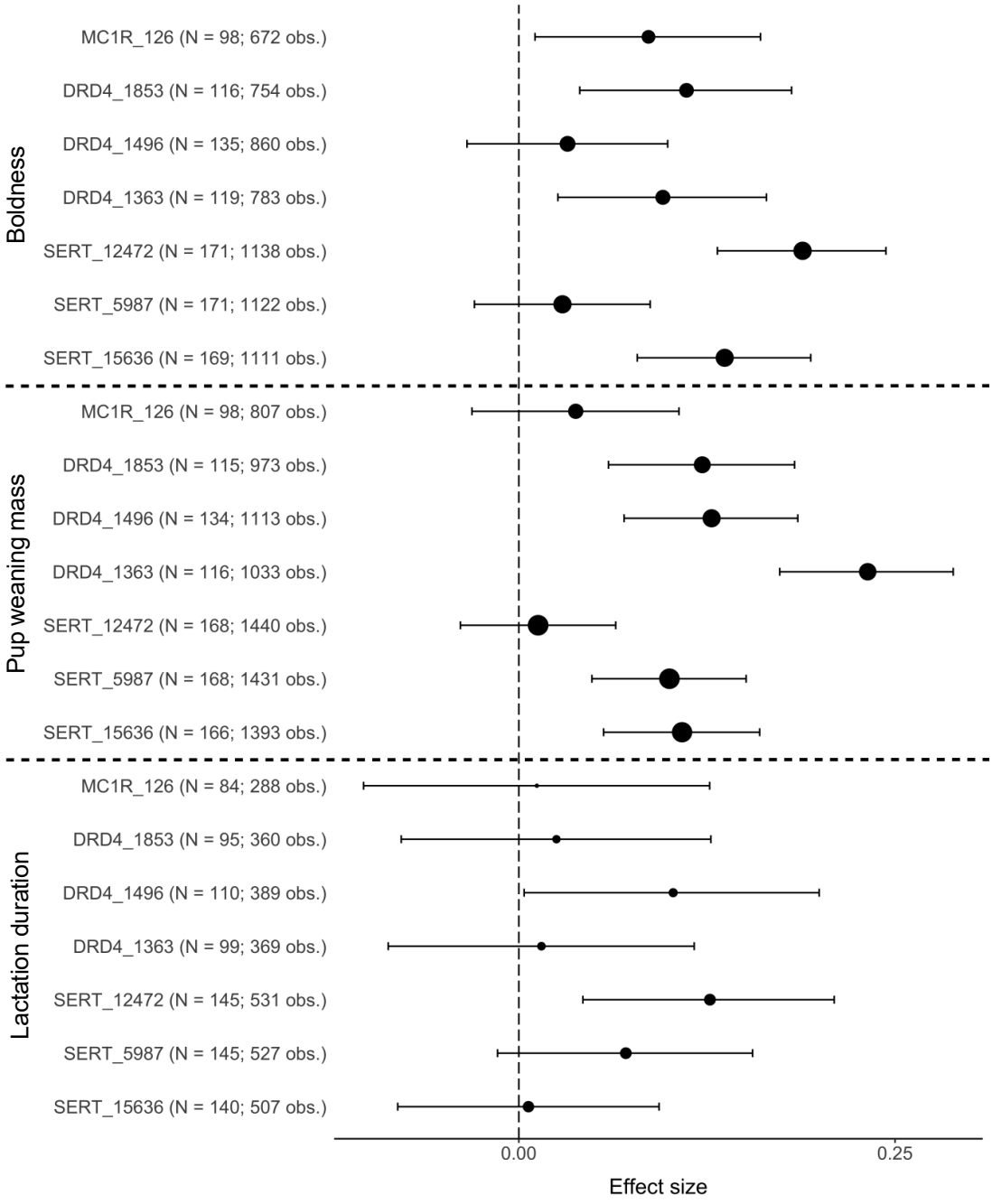

Figure 4.5: Forest plots of effect size estimates (± 95% confidence intervals) for the association of seven loci across three candidate genes with boldness, pup weaning mass, and lactation duration in female grey seals of Sable Island, Nova Scotia (Canada). Size of the point is proportional to the number of observations used in association tests.

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Appendices

C.1 Additional tables

Table C.1: Total number of females behaviourally tested and listed according to the female's year of birth (cohort).

Cohort	# of females
1974	3
1985	8
1986	13
1987	8
1989	7
1998	24
1999	33
2000	31
2001	25
2002	36

Table C.2: Description and number of boldness observations with respect to birth-site selection as characterized by microhabitat features.

Full Habitat Assessment				**Collapsed Habitat Assessment used for Modelling**			
Category	**Habitat**	**Tide/Flood**	**# of Obs.**	**Category**	**Habitat**	**Tide/Flood**	**# of Obs.**
1	Sand, shoreline	No	148	1	Sand inland and shoreline	No	393
2	Sand, shoreline	Yes	122	2	Sand or vegetated dunes	No	541
3	Sand, inland	No	245	3	Sand inland and shoreline	Yes	244
4	Sand, inland	Yes	122				
5	Dunes, vegetated	No	452				
6	Dunes, sand	No	72				
7	Hummocks	No	17				

Table C.3: Primers designed for targeting all or the majority of five candidate genes to examine the influence of genotype on boldness and maternal performance variation in grey seals of Sable Island, Nova Scotia (Canada). Refer to GenBank accession numbers MW864572-MW864597 and MW882063 for sequenced gene regions.

Primer pair name	5'-3'		Base pairs
DRD4-1 (2193_4F+2193_3R)	Forward	ACTCGGCGTTGAAGACAGTG	379
	Reverse	TGCAGGGAAGCAAAAGGAGG	
DRD4-2 (F1217+R1217)	Forward	ACAGGTTGAAGATGGAGGCG	335
	Reverse	CCAGGCCTTTCTCGGCTTTA	
DRD4-3 (F1302+R1715)	Forward	GCGAGATTCCCACCCAGAAA	414
	Reverse	TCAGCGGGAGGGGATTTAGA	
DRD4-4 (2194_4F+2194_1R)	Forward	TCTAAATCCCCTCCCGCTGA	1068
	Reverse	GACGCCCACCAACTACTTCA	
DRD4-5 (F2363+R2765)	Forward	AACCTCGCCTGTTTCTGAGG	422
	Reverse	CAGACGCCCACCAACTACTT	
OXTR-1	Forward	GAAAGGGTTCAGGGGTCCAG	1030
	Reverse	GATAGGCCCGTTACATGGGG	
OXTR-2	Forward	GAGGTGGGAGTTGTCGTGAG	558
	Reverse	AGGCAATCTCCCTGCTTGAC	
OXTR-3	Forward	CACCACTGTGGACTCCTGTC	210
	Reverse	GTGGGAGGAATTGGGACCAG	
OXTR-4	Forward	CTGCAAAGATGGCAACGCTT	507
	Reverse	ACCCAGCAAGTATCCAGTGC	
MC1R-1 (F7+R6)	Forward	GTCTCTTCAGCTGGGTCATG	1212

	Reverse	TCCACACCTCCGGAGATCAT	
MC5R-1	Forward	CTGCCAGGAAACCTACAGGG	839
	Reverse	ACTCTGGGTCTCATCAGCCT	
SERT 1 (F2774+R3527)	Forward	CTTTACTGGATGTCCATAACACG	754
	Reverse	ATGGCAGAAAGAGCTGGACC	
SERT 2 (F5040+R6452)	Forward	CACTCTGCTGCTTGACCTCA	1413
	Reverse	CACCCCAGCCCAAATGATCT	
SERT 3 (F6505+R7934)	Forward	GGATGCGGGATAATGAGGGG	890
	Reverse	ATTTCTAGCCCCAAGGTGCC	
SERT 4 (F7375+R8762)	Forward	GGCACCTTGGGGCTAGAAAT	1388
	Reverse	CCACCCAAAGCCACCAAATG	
SERT 5(F11013+R11817)	Forward	CTGGACACAGTCAGGAGCAG	805
	Reverse	CGCTGATAGCTCCTTCTGGG	
SERT 6 (F11751+R12338)	Forward	TCACCGTCATCTCCTAGCCA	607
	Reverse	GAGGTCAGGTTTGGGAGAGC	
SERT 7 (F12338+R13371)	Forward	GCTCTCCCAAACCTGACCTC	1034
	Reverse	GAAACCGGAAGTTGGGCAAC	
SERT 8 (15185F+15953R)	Forward	GCATTGTGTTGTGTGGGGAC	769
	Reverse	TCCAGGTGACGTTGTTCTCG	
SERT 9 (.1799F+.2982R)	Forward	ATCTCCTCCTTCACCGACCA	1090
	Reverse	AGACGTTTTGACACCCTTCCA	
SERT 10 (.5326F+.6543R)	Forward	GTGGTGAACTGCATGACGAG	1189
	Reverse	AGGCCCAGCGTGATTAACAT	
SERT 11 (F20704+R21937)	Forward	TGGGCTAATGAGGGCTAGGT	1234

	Reverse	GAATCTGGGCGAGTTCACCA	
SERT 12 (F22216+R23532)	Forward	CAGTGCATTGAGGGCCCTTA	1317
	Reverse	TGCCCATTCACATGCCAGAT	
SERT 13 (F23513+R24654)	Forward	ATCTGGCATGTGAATGGGCA	1032
	Reverse	GAAGGAGAGACACTTGCCCC	
SERT 14 (F26693+R27976)	Forward	AGCCTCCTCCCAGACTTAGG	1283
	Reverse	AGAGGAGATGAGTCCCCCAC	
SERT 15 (F29622+R30513)	Forward	GAGGTCAAGGTCAGGTGTGG	892
	Reverse	AGGGCCCATCTTCCCAGTAT	

Table C.4: Model selection for gene-phenotype associations for boldness, pup weaning mass, and lactation duration in grey seals of Sable Island. Akaike's Information Criterion (AIC) values for AIC, ΔAIC (difference in AIC from top model), and Akaike weight (*w*) are provided. Support for the top model given by lowest AIC, smallest ΔAIC, and highest *w*.

Model	AIC	ΔAIC	*w*
Boldness			
B ~ MC1R_126 + Age + Habitat + (1|BrandID) + (1|Year)	**1795.07**	**0**	**0.85**
B ~ MC1R_126 + Age + Habitat + (1|BrandID)	1798.5	3.43	0.15
B ~ MC1R_126 + Age + (1|BrandID) + (1|Year)	1817.66	22.59	0
B ~ MC1R_126 + Age + (1|BrandID)	1821.7	26.63	0
B ~ MC1R_126 + (1|BrandID)	1856.5	61.42	0
B ~ DRD4_1853 + DRD4_1496 + DRD4_1363 + Age + Habitat + (1|BrandID)	**1308.02**	**0**	**0.74**
B ~ DRD4_1853 + DRD4_1496 + DRD4_1363 + Age + Habitat + (1|BrandID) + (1|Year)	1310.13	2.11	0.26
B ~ DRD4_1853 + DRD4_1496 + DRD4_1363 + Age + (1|BrandID)	1318.6	10.58	0
B ~ DRD4_1853 + DRD4_1496 + DRD4_1363 + Age + (1|BrandID) + (1|Year)	1320.69	12.68	0
B ~ DRD4_1853 + DRD4_1496 + DRD4_1363 + (1|BrandID)	1340.27	32.25	0
B ~ SERT_12472 + SERT_5987 + SERT_15636 + Age + Habitat + (1|BrandID) + (1|Year)	**2739.598**	**0**	**0.791**
B ~ SERT_12472 + SERT_5987 + SERT_15636 + Age + Habitat + (1|BrandID)	2742.26	2.6621	0.209
B ~ SERT_12472 + SERT_5987 + SERT_15636 + Age + (1|BrandID) + (1|Year)	2772.496	32.8982	0
B ~ SERT_12472 + SERT_5987 + SERT_15636 + Age + (1|BrandID)	2775.3	35.7016	0

B ~ SERT_12472 + SERT_5987 + SERT_15636 + (1|BrandID)	2812.837	73.2392	0
Pup Weaning Mass (pwm)			
pwm ~ MC1R_126 + Age + Sex + (1|BrandID) + (1|Year)	**5263.09**	**0**	**1**
pwm ~ MC1R_126 + Age + Sex + (1|BrandID)	5311.66	48.57	0
pwm ~ DRD4_1853 + DRD4_1496 + DRD4_1363 + Age + Sex + (1|BrandID) + (1|Year)	**4384.62**	**0**	**1**
pwm ~ DRD4_1853 + DRD4_1496 + DRD4_1363 + Age + Sex + (1|BrandID)	4410.02	25.39	0
pwm ~ SERT_12472 + SERT_5987 + SERT_15636 + Age + Sex + (1|BrandID) + (1|Year)	**8364.29**	**0**	**1**
pwm ~ SERT_12472 + SERT_5987 + SERT_15636 + Age + Sex + (1|BrandID)	8453.51	89.22	0
Lactation Duration (LD)			
LD ~ MC1R_126 + Age + (1|BrandID)	1292.17	0	0.74
LD ~ MC1R_126 + Age + (1|BrandID) + (1|Year)	**1294.3**	**2.13**	**0.26**
LD ~ DRD4_1853 + DRD4_1496 + DRD4_1363 + Age + (1|BrandID) + (1|Year)	**1073.98**	**0**	**0.56**
LD ~ DRD4_1853 + DRD4_1496 + DRD4_1363 + Age + (1|BrandID)	1074.46	0.48	0.44
LD ~ SERT_12472 + SERT_5987 + SERT_15636 + Age + (1|BrandID)	2114.88	0	0.59
LD ~ SERT_12472 + SERT_5987 + SERT_15636 + Age + (1|BrandID) + (1|Year)	**2115.64**	**0.76**	**0.41**

(1|BrandID) and (1|Year) represent the random factors included in models.

*Bolded models indicate preferred models used for analyses.

Table C.5: Genetic diversity indices and Tajima's *D* neutrality test estimates for each candidate gene region sequenced. k is the average number of pairwise nucleotide differences within the population. Bolded values indicate significance at $P < 0.05$.

Gene region	# of SNPs	k	Nucleotide diversity (π)	Tajima's *D*
DRD4-1	0	0	0.000	NA
DRD4-2	0	0	0.000	NA
DRD4-3	2	0.26407	0.0007	-1.175
DRD4-4	0	0	0.000	NA
DRD4-5	1	0.44211	0.00095	1.026
MC1R	3	0.72857	0.00082	0.016
MC5R	1	0.08502	0.00011	-0.860
OXTR-1	2	0.3372	0.00039	-0.472
OXTR-2	1	0.2139	0.00049	-0.188
OXTR-3	0	0	0.000	NA
OXTR-4	0	0	0.000	NA
SERT-1	1	0.51494	0.00082	1.621
SERT-2	4	1.93116	0.0036	**2.196**
SERT-3	3	1.06667	0.00159	0.021
SERT-4	4	1.29167	0.00159	0.224
SERT-5	3	0.86667	0.00139	-0.120
SERT-6	0	0	0.000	NA
SERT-7	1	0.50327	0.00073	1.378
SERT-8	5	1.85185	0.00628	1.226
SERT-9	1	0.39829	0.00079	0.976
SERT-10	1	0.05795	0.00007	-0.900
SERT-11	1	0.12874	0.00035	-0.764
SERT-12	0	0	0.000	NA
SERT-13	0	0	0.000	NA
SERT-14	1	0.05882	0.00009	-1.138
SERT-15	1	0.39605	0.00103	0.992

Table C.6: Matrix of FDR-corrected P values from linkage disequilibrium analysis of *SERT* loci.

	S5987	**S12472**	**S2946**	**S6056**	**S15549**	**S11673**	**S11432**	**S15636**	**S16657**	**S7535**	**S7540**	**S11689**
S12472	0.8155											
S2946	0	0.9599										
S6056	0	0.9992	0									
S15549	0	0.931	0	0								
S11673	0.0044	1	0.0001	0.0001	0.003							
S11432	0.0001	0.8262	0.0004	0	0.0098	0.5621						
S15636	0.8523	0.3004	0.9004	0.5621	0.2767	0.8424	0.2767					
S16657	0.0007	0.271	0.0001	0.0014	0.0032	0.0175	0	0.8424				
S7535	0	0.8424	0	0	0	0.0028	0.0002	0.294	0.0005			
S7540	0	0.3924	0	0	0	0.0107	0.0046	0.9138	0.0272	0		
S11689	0.6861	0.8155	0.8424	0.3004	0.8424	0.5601	0.7876	0.9004	0.5565	0.8155	1	
S6147	0	0.8523	0	0	0	0.0002	0.0001	0.931	0.0008	0	0	0.7

Table C.7: Full additive model results, including covariates age, habitat, and pup sex, from preferred models assessing gene-phenotype associations for boldness, pup weaning mass (PWM), and lactation duration in female grey seals of Sable Island. Female identity and year were included in models as random factors.

	Fixed factors	Estimate	SE	df	t	P-value	2.5% CI	97.5% CI	Effect size (r)
Boldness ~ SNP(s) + Age + Habitat; 180 females, 1183 total observations									
MC1R	**(Intercept)**	**2.978**	**0.288**	**117.929**	**10.345**	**< 0.0001**	**2.408**	**3.551**	**0.690**
	*MC1R*_126	-0.193	0.226	97.983	-0.856	0.394	-0.641	0.254	0.086
	Age 11-15 years	**0.224**	**0.096**	**65.588**	**2.333**	**0.0227**	**0.019**	**0.414**	**0.277**
	Age 16-20 years	**0.472**	**0.125**	**25.914**	**3.778**	**< 0.001**	**0.183**	**0.718**	**0.596**
	Age 21-25 years	**1.300**	**0.355**	**120.511**	**3.657**	**< 0.001**	**0.597**	**2.001**	**0.316**
	Age ≥ 26 years	**1.321**	**0.366**	**131.181**	**3.615**	**< 0.001**	**0.596**	**2.044**	**0.301**
	Dunes	**0.297**	**0.079**	**601.476**	**3.773**	**< 0.001**	**0.143**	**0.452**	**0.152**
	Sand (flood)	0.116	0.087	583.592	1.344	0.179	-0.054	0.287	0.056
DRD4	**(Intercept)**	**3.040**	**0.422**	**88.629**	**7.204**	**< 0.0001**	**2.205**	**3.877**	**0.608**
	*DRD4*_1853	-0.197	0.202	75.629	-0.975	0.333	-0.599	0.203	0.111
	*DRD4*_1496	-0.073	0.255	77.416	-0.285	0.776	-0.579	0.435	0.0324
	*DRD4*_1363	0.255	0.307	74.517	0.830	0.409	-0.355	0.864	0.0957
	Age 11-15 years	**0.210**	**0.097**	**420.679**	**2.178**	**0.0299**	**0.021**	**0.400**	**0.106**
	Age 16-20 years	**0.523**	**0.108**	**427.585**	**4.859**	**< 0.0001**	**0.311**	**0.734**	**0.229**
	Age 21-25 years	**0.800**	**0.272**	**343.584**	**2.944**	**0.00346**	**0.262**	**1.334**	**0.157**
	Age ≥ 26 years	**1.041**	**0.291**	**373.185**	**3.582**	**< 0.001**	**0.466**	**1.612**	**0.182**
	Dunes	**0.264**	**0.086**	**431.212**	**3.063**	**0.00233**	**0.095**	**0.433**	**0.146**
	Sand (flood)	*0.206*	*0.106*	*432.461*	*1.944*	*0.0526*	*-0.00258*	*0.413*	*0.0931*
SERT	**(Intercept)**	**1.979**	**0.558**	**156.060**	**3.544**	**< 0.0001**	**0.878**	**3.081**	**0.273**
	***SERT*_12472**	**0.626**	**0.267**	**149.329**	**2.346**	**0.020**	**0.100**	**1.152**	**0.189**
	*SERT*_5987	0.049	0.138	151.509	0.357	0.722	-0.222	0.320	0.029

	SERT_15636	*-0.303*	*0.180*	*149.401*	*-1.688*	*0.093*	*-0.657*	*0.051*	*0.137*
	Age 11-15 years	**0.283**	**0.080**	**95.747**	**3.542**	**< 0.001**	**0.124**	**0.440**	**0.340**
	Age 16-20 years	**0.500**	**0.099**	**35.530**	**5.073**	**< 0.0001**	**0.297**	**0.696**	**0.648**
	Age 21-25 years	**0.805**	**0.218**	**447.401**	**3.693**	**< 0.001**	**0.373**	**1.233**	**0.172**
	Age ≥ 26 years	**0.851**	**0.234**	**241.606**	**3.633**	**< 0.001**	**0.384**	**1.312**	**0.228**
	Dunes	**0.283**	**0.063**	**902.680**	**4.467**	**< 0.0001**	**0.159**	**0.408**	**0.147**
	Sand (flood)	0.089	0.072	886.787	1.223	0.222	-0.054	0.231	0.041
PWM ~ SNP(s) + Age + Sex; 177 females, 1496 total observations									
MC1R	**(Intercept)**	**47.503**	**1.480**	**117.636**	**32.089**	**< 0.0001**	**44.560**	**50.417**	**0.947**
	*MC1R*_126	0.381	1.062	90.016	0.359	0.721	-1.716	2.498	0.038
	Age 11-15 years	**5.983**	**0.686**	**280.710**	**8.719**	**< 0.0001**	**4.547**	**7.371**	**0.462**
	Age 16-20 years	**7.322**	**0.875**	**178.878**	**8.371**	**< 0.0001**	**5.446**	**9.096**	**0.531**
	Age 21-25 years	**6.752**	**1.179**	**282.079**	**5.729**	**< 0.0001**	**4.213**	**9.168**	**0.323**
	Age ≥ 26 years	**3.525**	**1.503**	**181.959**	**2.346**	**0.0201**	**0.178**	**6.637**	**0.171**
	Sex (female)	**-2.060**	**0.406**	**704.383**	**-5.078**	**< 0.0001**	**-2.856**	**-1.264**	**0.188**
DRD4	**(Intercept)**	**49.416**	**1.578**	**86.415**	**31.307**	**< 0.0001**	**46.275**	**52.572**	**0.959**
	*DRD4*_1853	-0.726	0.730	65.650	-0.995	0.323	-2.178	0.726	0.122
	*DRD4*_1496	-0.984	0.924	68.000	-1.065	0.291	-2.828	0.857	0.128
	DRD4_1363	*2.274*	*1.146*	*69.383*	*1.985*	*0.051*	*0.00399*	*4.561*	*0.232*
	Age 11-15 years	**6.598**	**0.718**	**295.348**	**9.197**	**< 0.0001**	**5.119**	**8.035**	**0.472**
	Age 16-20 years	**7.545**	**0.848**	**176.815**	**8.902**	**< 0.0001**	**5.709**	**9.269**	**0.556**
	Age 21-25 years	**6.874**	**1.169**	**272.879**	**5.878**	**< 0.0001**	**4.354**	**9.264**	**0.335**
	Age ≥ 26 years	**2.995**	**1.503**	**190.006**	**1.992**	**0.0478**	**-0.326**	**6.088**	**0.143**
	Sex (female)	**-2.099**	**0.464**	**588.253**	**-4.527**	**< 0.0001**	**-3.009**	**-1.189**	**0.183**
SERT	**(Intercept)**	**45.347**	**2.533**	**144.528**	**17.902**	**< 0.0001**	**40.350**	**50.349**	**0.830**
	*SERT*_12472	0.177	1.196	133.524	0.148	0.883	-2.184	2.538	0.013
	*SERT*_5987	0.722	0.620	133.945	1.164	0.246	-0.502	1.945	0.100
	*SERT*_15636	1.002	0.802	131.035	1.249	0.214	-0.587	2.583	0.108
	Age 11-15 years	**6.107**	**0.553**	**549.739**	**11.039**	**< 0.0001**	**4.936**	**7.245**	**0.426**

	Age 16-20 years	**7.116**	**0.693**	**271.357**	**10.263**	**< 0.0001**	**5.572**	**8.563**	**0.529**
	Age 21-25 years	**5.251**	**0.927**	**302.600**	**5.666**	**< 0.0001**	**3.128**	**7.224**	**0.310**
	Age ≥ 26 years	1.365	1.193	195.935	1.144	0.254	-1.463	3.936	0.081
	Sex (female)	**-2.349**	**0.327**	**1123.808**	**-7.193**	**< 0.0001**	**-2.989**	**-1.708**	**0.210**
Lactation duration ~ SNP(s) + Age; 151 females, 542 total observations									
MC1R	**(Intercept)**	**15.629**	**0.408**	**65.749**	**38.293**	**< 0.0001**	**14.823**	**16.473**	**0.978**
	*MC1R*_126	-0.028	0.291	65.567	-0.097	0.923	-0.615	0.545	0.012
	Age 11-15 years	**2.035**	**0.315**	**61.799**	**6.454**	**< 0.0001**	**1.388**	**2.655**	**0.635**
	Age 16-20 years	**2.855**	**0.359**	**97.156**	**7.943**	**< 0.0001**	**2.145**	**3.569**	**0.627**
	Age 21-25 years	**1.844**	**0.526**	**281.229**	**3.505**	**< 0.0001**	**0.805**	**2.885**	**0.205**
	Age ≥ 26 years	**2.932**	**0.861**	**249.042**	**3.405**	**0.00077**	**1.236**	**4.625**	**0.211**
DRD4	**(Intercept)**	**16.498**	**0.493**	**67.510**	**33.476**	**< 0.0001**	**15.525**	**17.491**	**0.971**
	*DRD4*_1853	0.043	0.233	55.322	0.186	0.853	-0.420	0.510	0.025
	*DRD4*_1496	-0.267	0.308	70.740	-0.867	0.389	-0.878	0.346	0.103
	*DRD4*_1363	0.053	0.401	78.421	0.133	0.894	-0.744	0.850	0.015
	Age 11-15 years	**1.799**	**0.325**	**125.248**	**5.533**	**< 0.0001**	**1.115**	**2.456**	**0.443**
	Age 16-20 years	**2.305**	**0.362**	**146.331**	**6.371**	**< 0.0001**	**1.585**	**3.022**	**0.466**
	Age 21-25 years	**1.839**	**0.528**	**242.650**	**3.483**	**< 0.0001**	**0.782**	**2.895**	**0.218**
	Age ≥ 26 years	*2.127*	*1.154*	*218.695*	*1.844*	*0.0666*	*-0.155*	*4.400*	*0.124*
SERT	**(Intercept)**	**14.688**	**0.701**	**133.107**	**20.956**	**< 0.0001**	**13.310**	**16.088**	**0.876**
	*SERT*_12472	0.469	0.337	118.074	1.391	0.167	-0.205	1.131	0.127
	*SERT*_5987	0.133	0.172	117.297	0.772	0.442	-0.207	0.477	0.071
	*SERT*_15636	0.015	0.224	116.217	0.069	0.945	-0.430	0.456	0.006
	Age 11-15 years	**1.975**	**0.249**	**109.032**	**7.926**	**< 0.0001**	**1.463**	**2.466**	**0.605**
	Age 16-20 years	**2.676**	**0.277**	**164.687**	**9.658**	**< 0.0001**	**2.132**	**3.229**	**0.601**
	Age 21-25 years	**1.991**	**0.391**	**444.971**	**5.098**	**< 0.0001**	**1.223**	**2.759**	**0.235**
	Age ≥ 26 years	**2.378**	**0.571**	**379.695**	**4.164**	**< 0.0001**	**1.255**	**3.506**	**0.209**

*Bolded values indicate statistically significant terms (P <0.05). Italicized values indicated marginally non-significant terms (P <0.10).

Table C.8: Estimates of the proportion of trait variation [boldness, pup weaning mass (PWM), and lactation duration (LD)] explained by each gene, SNP, and other fixed factors (i.e. age, habitat, and pup sex), as well as all loci combined, calculated from marginal R^2 ($R^2_{GLMM(m)}$). Female identity and year were included in models as random factors. Conditional R^2 ($R^2_{GLMM(c)}$) also provided.

	$R^2_{GLMM(m)}$	$R^2_{GLMM(c)}$
Boldness ~ SNP(s) + Age + Habitat		
MC1R	9.48E-05	0.734
DRD4	0.0199	0.747
*DRD4*_1853	0.0038	0.745
*DRD4*_1496	0.0002	0.730
*DRD4*_1363	0.0047	0.712
SERT	0.0345	0.739
*SERT*_12472	0.0209	0.738
*SERT*_5987	0.0038	0.730
*SERT*_15636	0.0133	0.739
All Loci	0.1366	0.751
Age	0.0285	0.726
Habitat	0.0057	0.737
Null (only random)	0	0.733
Total:	0.2056	0.763
PWM ~ SNP(s) + Age + Sex		
MC1R	0.00125	0.598
DRD4	0.01781	0.502
*DRD4*_1853	0.00116	0.531
*DRD4*_1496	0.0008	0.526
*DRD4*_1363	0.00472	0.555
SERT	0.00648	0.534
*SERT*_12472	0.00116	0.542
*SERT*_5987	0.00289	0.538

*SERT*_15636	0.00381	0.545
All Loci	0.0652	0.449
Age	0.1224	0.554
Pup sex	0.0206	0.567
Null (only random)	0	0.544
Total:	0.277	0.512
LD ~ SNP(s) + Age + Sex		
MC1R	0.00014	0.289
DRD4	0.00977	0.411
*DRD4*_1853	0.00153	0.346
*DRD4*_1496	0.00039	0.328
*DRD4*_1363	0.00020	0.386
SERT	0.00677	0.300
*SERT*_12472	0.00197	0.330
*SERT*_5987	0.00460	0.337
*SERT*_15636	0.00031	0.306
All Loci	0.106	0.330
Age	0.188	0.340
Null (only random)	0	0.333
Total:	0.331	0.367

Table C.9: Overdominance (OD) model results for the genetic effect on boldness in female grey seals of Sable Island, Nova Scotia (Canada). Results are based on a sample size of up to 180 females and 1183 observations over an 11-year period. Female identity and year were included in models as random factors.

	Fixed factors	Estimate	SE	df	t	P-value	2.5% CI	97.5% CI	Effect size (r)
Boldness ~ OD SNP(s) + Age + Habitat									
MC1R	**(Intercept)**	**2.839**	**0.177**	**127.197**	**16.084**	**< 0.0001**	**2.491**	**3.194**	**0.819**
	126_Homozyg	-0.228	0.261	98.806	-0.873	0.385	-0.745	0.288	0.087
	Age 11-15 years	**0.228**	**0.096**	**66.024**	**2.368**	**0.021**	**0.023**	**0.417**	**0.280**
	Age 16-20 years	**0.477**	**0.125**	**26.083**	**3.824**	**< 0.0001**	**0.190**	**0.723**	**0.599**
	Age 21-25 years	**1.259**	**0.347**	**122.721**	**3.628**	**< 0.001**	**0.573**	**1.945**	**0.311**
	Age > 26 years	**1.282**	**0.358**	**134.522**	**3.579**	**< 0.001**	**0.571**	**1.989**	**0.295**
	Dunes	**0.296**	**0.079**	**601.075**	**3.755**	**< 0.001**	**0.141**	**0.451**	**0.151**
	Sand (flood)	0.116	0.087	583.742	1.339	0.181	-0.054	0.286	0.055
DRD4	**(Intercept)**	**3.031**	**0.399**	**85.177**	**7.592**	**< 0.0001**	**2.241**	**3.824**	**0.635**
	1853_Homozyg	0.078	0.297	76.573	0.262	0.794	-0.509	0.669	0.030
	1496_Homozyg	0.348	0.290	77.322	1.203	0.233	-0.227	0.923	0.136
	1363_Homozyg	-0.468	0.376	76.367	-1.244	0.217	-1.215	0.278	0.141
	Age 11-15 years	**0.210**	**0.097**	**420.815**	**2.175**	**0.030**	**0.020**	**0.400**	**0.105**
	Age 16-20 years	**0.525**	**0.108**	**427.812**	**4.886**	**< 0.0001**	**0.314**	**0.737**	**0.230**
	Age 21-25 years	**0.846**	**0.273**	**346.180**	**3.103**	**0.00207**	**0.305**	**1.382**	**0.165**
	Age > 26 years	**1.093**	**0.292**	**379.155**	**3.741**	**< 0.001**	**0.513**	**1.669**	**0.189**

	Dunes	**0.262**	**0.086**	**431.510**	**3.049**	**0.00244**	**0.093**	**0.431**	**0.145**
	Sand (flood)	*0.207*	*0.106*	*432.551*	*1.953*	*0.0515*	*-0.002*	*0.414*	*0.093*
SERT	**(Intercept)**	**2.191**	**0.309**	**167.209**	**7.103**	**< 0.0001**	**1.584**	**2.801**	**0.481**
	12472_Homozyg	*0.528*	*0.289*	*148.154*	*1.825*	*0.070*	*-0.042*	*1.098*	*0.148*
	5987_Homozyg	0.263	0.203	153.217	1.296	0.197	-0.138	0.663	0.104
	15636_Homozyg	-0.060	0.201	152.063	-0.299	0.765	-0.455	0.336	0.024
	Age 11-15 years	**0.282**	**0.080**	**95.500**	**3.527**	**< 0.0001**	**0.123**	**0.439**	**0.339**
	Age 16-20 years	**0.498**	**0.099**	**35.434**	**5.046**	**< 0.0001**	**0.294**	**0.693**	**0.647**
	Age 21-25 years	**0.839**	**0.220**	**452.459**	**3.816**	**< 0.001**	**0.402**	**1.270**	**0.177**
	Age > 26 years	**0.886**	**0.236**	**242.600**	**3.758**	**< 0.0001**	**0.415**	**1.351**	**0.235**
	Dunes	**0.284**	**0.063**	**902.965**	**4.486**	**< 0.0001**	**0.160**	**0.409**	**0.148**
	Sand (flood)	0.090	0.072	886.603	1.246	0.213	-0.052	0.233	0.042

*Bolded values indicate statistical significance ($P < 0.05$). Italicized values are marginally non-significant ($P < 0.10$)

Table C.10: Overdominance (OD) model results for the genetic effect on pup weaning mass in female grey seals of Sable Island, Nova Scotia (Canada). Results are based on a sample size of up to 177 females and 1496 observations over a 28-year period. Female identity and year were included in models as random factors.

	Fixed factors	Estimate	SE	df	t	P-value	2.5% CI	97.5% CI	Effect size (r)
PWM ~ OD SNP(s) + Age + Sex									
MC1R	**(Intercept)**	**47.748**	**0.956**	**111.892**	**49.924**	**< 0.0001**	**45.856**	**49.643**	**0.978**
	126_Homozyg	0.581	1.253	89.294	0.464	0.644	-1.896	3.073	0.049
	Age 11-15 years	**5.978**	**0.686**	**281.062**	**8.709**	**< 0.0001**	**4.542**	**7.366**	**0.461**
	Age 16-20 years	**7.317**	**0.875**	**179.611**	**8.362**	**< 0.0001**	**5.441**	**9.090**	**0.529**
	Age 21-25 years	**6.761**	**1.176**	**287.227**	**5.75**	**< 0.0001**	**4.231**	**9.170**	**0.321**
	Age > 26 years	**3.537**	**1.500**	**184.727**	**2.358**	**0.0194**	**0.202**	**6.643**	**0.171**
	Sex (female)	**-2.056**	**0.406**	**704.551**	**-5.068**	**< 0.0001**	**-2.853**	**-1.260**	**0.188**
DRD4	**(Intercept)**	**47.486**	**1.611**	**85.105**	**29.485**	**< 0.0001**	**44.291**	**50.678**	**0.954**
	1853_Homozyg	*1.877*	*1.097*	*67.188*	*1.711*	*0.092*	*-0.303*	*4.069*	*0.204*
	1496_Homozyg	0.880	1.077	68.291	0.817	0.417	-1.257	3.032	0.098
	1363_Homozyg	-1.086	1.430	69.054	-0.759	0.450	-3.938	1.749	0.091
	Age 11-15 years	**6.587**	**0.718**	**294.530**	**9.17**	**< 0.0001**	**5.105**	**8.026**	**0.471**
	Age 16-20 years	**7.511**	**0.850**	**175.545**	**8.839**	**< 0.0001**	**5.669**	**9.239**	**0.555**
	Age 21-25 years	**6.842**	**1.176**	**265.600**	**5.817**	**< 0.0001**	**4.303**	**9.248**	**0.336**
	Age > 26 years	*2.966*	*1.513*	*184.934*	*1.96*	*0.052*	*-0.384*	*6.081*	*0.143*
	Sex (female)	**-2.103**	**0.463**	**587.939**	**-4.539**	**< 0.0001**	**-3.014**	**-1.194**	**0.184**

SERT	**(Intercept)**	**47.684**	**1.433**	**147.651**	**33.267**	**< 0.0001**	**44.859**	**50.521**	**0.939**
	12472_Homozyg	0.282	1.292	129.937	0.219	0.827	-2.269	2.833	0.019
	5987_Homozyg	-0.429	0.909	133.732	-0.472	0.637	-2.229	1.363	0.041
	15636_Homozyg	0.600	0.902	133.447	0.665	0.507	-1.185	2.376	0.057
	Age 11-15 years	**6.088**	**0.554**	**545.908**	**10.985**	**< 0.0001**	**4.910**	**7.230**	**0.425**
	Age 16-20 years	**7.071**	**0.696**	**264.416**	**10.163**	**< 0.0001**	**5.510**	**8.530**	**0.530**
	Age 21-25 years	**5.150**	**0.932**	**291.685**	**5.529**	**< 0.0001**	**3.000**	**7.143**	**0.308**
	Age > 26 years	1.236	1.200	189.414	1.03	0.304	-1.633	3.837	0.075
	Sex (female)	**-2.354**	**0.327**	**1121.639**	**-7.211**	**< 0.0001**	**-2.995**	**-1.714**	**0.210**

*Bolded values indicate statistical significance ($P < 0.05$). Italicized values are marginally non-significant ($P < 0.10$).

Table C.11: Overdominance (OD) model results for the genetic effect on lactation duration in female grey seals of Sable Island, Nova Scotia (Canada). Results are based on a sample size of up to 151 females and 542 observations over a 28-year period. Female identity and year were included in models as random factors.

	Fixed factors	Estimate	SE	df	t	P-value	2.5% CI	97.5% CI	Effect size (r)
Lactation duration ~ OD SNP(s) + Age									
MC1R	**(Intercept)**	**15.648**	**0.257**	**41.215**	**60.856**	**< 0.0001**	**15.141**	**16.195**	**0.994**
	126_Homozyg	-0.1519	0.350	69.658	-0.434	0.666	-0.852	0.541	0.052
	Age 11-15 years	**2.0445**	**0.316**	**62.777**	**6.471**	**< 0.0001**	**1.397**	**2.666**	**0.633**
	Age 16-20 years	**2.8585**	**0.359**	**96.930**	**7.954**	**< 0.0001**	**2.148**	**3.572**	**0.628**
	Age 21-25 years	**1.852**	**0.525**	**281.384**	**3.531**	**< 0.0001**	**0.815**	**2.890**	**0.206**
	Age > 26 years	**2.9099**	**0.862**	**251.736**	**3.376**	**< 0.001**	**1.212**	**4.605**	**0.208**
DRD4	**(Intercept)**	**15.717**	**0.542**	**78.923**	**29**	**< 0.0001**	**14.651**	**16.817**	**0.956**
	1853_Homozyg	0.251	0.349	57.001	0.719	0.475	-0.449	0.942	0.095
	1496_Homozyg	0.249	0.352	68.141	0.706	0.483	-0.460	0.945	0.085
	1363_Homozyg	0.276	0.471	61.757	0.586	0.560	-0.672	1.215	0.074
	Age 11-15 years	**1.823**	**0.326**	**127.950**	**5.585**	**< 0.0001**	**1.138**	**2.482**	**0.443**
	Age 16-20 years	**2.342**	**0.364**	**146.366**	**6.444**	**< 0.0001**	**1.619**	**3.064**	**0.470**
	Age 21-25 years	**1.904**	**0.532**	**241.423**	**3.579**	**< 0.0001**	**0.837**	**2.972**	**0.224**
	Age > 26 years	*2.177*	*1.157*	*218.099*	*1.882*	*0.0611*	*-0.109*	*4.456*	*0.126*
SERT	**(Intercept)**	**15.243**	**0.361**	**111.023**	**42.248**	**< 0.0001**	**14.533**	**15.965**	**0.970**
	12472_Homozyg	*0.678*	*0.350*	*112.146*	*1.936*	*0.055*	*-0.022*	*1.368*	*0.180*

5987_Homozyg	-0.142	0.238	102.077	-0.598	0.551	-0.613	0.330	0.059
15636_Homozyg	-0.026	0.239	104.639	-0.11	0.912	-0.498	0.450	0.011
Age 11-15 years	**1.983**	**0.247**	**104.472**	**8.034**	**< 0.0001**	**1.477**	**2.469**	**0.618**
Age 16-20 years	**2.668**	**0.275**	**158.969**	**9.698**	**< 0.0001**	**2.127**	**3.215**	**0.610**
Age 21-25 years	**1.981**	**0.390**	**444.063**	**5.082**	**< 0.0001**	**1.214**	**2.746**	**0.234**
Age > 26 years	**2.317**	**0.571**	**372.133**	**4.056**	**< 0.0001**	**1.194**	**3.444**	**0.206**

*Bolded values indicate statistical significance ($P < 0.05$). Italicized values are marginally non-significant ($P < 0.1$).

Table C.12: Additive model results using alternate loci from *SERT* linkage group (loci approaching significance and linked with *SERT*_5987). Alternate loci demonstrated similar effect sizes to those presented for association between *SERT*_5987 and boldness, pup weaning mass (PWM), and lactation duration. Female identity and year were included in models as random factors.

	Fixed factors	Estimate	SE	df	t	P	2.5% CI	97.5% CI	effect size	$R^2_{GLMM(m)}$
Boldness ~ SNP + Age + Habitat										0.0776
SERT	**(Intercept)**	**2.196**	**0.522**	**154.886**	**4.21**	**< 0.0001**	**1.167**	**3.225**	**0.320**	
	***SERT*_12472**	**0.597**	**0.252**	**148.204**	**2.364**	**0.0194**	**0.099**	**1.095**	**0.191**	
	SERT_15636	-0.300	0.181	149.228	-1.659	0.0992	-0.656	0.057	0.135	
	**SERT*_2946*	-0.088	0.141	151.755	-0.622	0.535	-0.365	0.190	0.050	0.00616
	AgeGroup2	**0.278**	**0.077**	**80.503**	**3.61**	**< 0.001**	**0.124**	**0.429**	**0.373**	
	AgeGroup3	**0.497**	**0.093**	**30.790**	**5.36**	**< 0.0001**	**0.308**	**0.683**	**0.695**	
	AgeGroup4	**0.798**	**0.225**	**481.228**	**3.552**	**< 0.001**	**0.353**	**1.239**	**0.160**	
	AgeGroup5	**0.880**	**0.237**	**250.662**	**3.707**	**< 0.001**	**0.408**	**1.347**	**0.228**	
	Habitat2	**0.261**	**0.063**	**919.148**	**4.15**	**< 0.0001**	**0.137**	**0.384**	**0.136**	
	Habitat3	0.092	0.072	902.715	1.277	0.202	-0.050	0.234	0.042	
Boldness ~ SNP + Age + Habitat										0.0913
SERT	**(Intercept)**	**2.182**	**0.564**	**129.719**	**3.868**	**< 0.001**	**1.068**	**3.297**	**0.322**	
	*SERT*_12472	*0.500*	*0.284*	*125.646*	*1.762*	*0.0805*	*-0.060*	*1.060*	*0.155*	
	SERT_15636	**-0.377**	**0.190**	**125.743**	**-1.985**	**0.0493**	**-0.752**	**-0.001**	**0.174**	
	**SERT*_6147*	0.023	0.151	128.126	0.152	0.879	-0.275	0.321	0.013	0.00085
	AgeGroup2	**0.380**	**0.078**	**70.753**	**4.9**	**< 0.0001**	**0.228**	**0.538**	**0.503**	

	AgeGroup3	**0.606**	**0.089**	**29.141**	**6.794**	**< 0.0001**	**0.430**	**0.796**	**0.783**	
	AgeGroup4	**0.950**	**0.218**	**426.703**	**4.353**	**< 0.0001**	**0.515**	**1.381**	**0.206**	
	AgeGroup5	**1.057**	**0.228**	**214.219**	**4.637**	**< 0.0001**	**0.602**	**1.512**	**0.302**	
	Habitat2	**0.204**	**0.065**	**769.568**	**3.139**	**0.0018**	**0.076**	**0.331**	**0.112**	
	Habitat3	0.0180	0.0748	761.188	0.241	0.810	-0.129	0.165	0.009	
PWM ~ SNP + Age + Sex										0.154
SERT	**(Intercept)**	**45.446**	**2.410**	**140.147**	**18.857**	**< 0.0001**	**40.694**	**50.208**	**0.847**	
	*SERT*_12472	0.863	1.149	128.835	0.751	0.454	-1.407	3.131	0.066	
	**SERT*_6056	-0.280	0.667	131.594	-0.419	0.676	-1.599	1.036	0.037	0.00041
	SERT_15636	0.767	0.842	128.162	0.911	0.364	-0.900	2.425	0.080	
	AgeGroup2	**6.067**	**0.568**	**528.356**	**10.691**	**< 0.0001**	**4.867**	**7.233**	**0.422**	
	AgeGroup3	**7.259**	**0.703**	**249.985**	**10.332**	**< 0.0001**	**5.693**	**8.723**	**0.547**	
	AgeGroup4	**5.261**	**0.938**	**287.007**	**5.608**	**< 0.0001**	**3.114**	**7.254**	**0.314**	
	AgeGroup5	**1.407**	**1.208**	**183.732**	**1.165**	**0.246**	**-1.453**	**4.002**	**0.086**	
	Sex (Female)	**-2.373**	**0.331**	**1104.106**	**-7.175**	**< 0.0001**	**-3.021**	**-1.724**	**0.211**	
PWM ~ SNP + Age + Sex										0.164
SERT	**(Intercept)**	**45.925**	**2.684**	**131.129**	**17.109**	**< 0.0001**	**40.614**	**51.215**	**0.831**	
	*SERT*_12472	0.232	1.136	118.956	0.204	0.8385	-2.009	2.481	0.019	
	**SERT*_7535	0.232	0.677	122.495	0.343	0.7325	-1.105	1.570	0.031	0.00054
	*SERT*_15636	0.999	0.819	117.772	1.22	0.225	-0.622	2.615	0.112	
	AgeGroup2	**6.485**	**0.566**	**477.418**	**11.451**	**< 0.0001**	**5.285**	**7.645**	**0.464**	
	AgeGroup3	**7.158**	**0.707**	**239.785**	**10.118**	**< 0.0001**	**5.564**	**8.640**	**0.547**	

	AgeGroup4	**6.180**	**0.960**	**278.376**	**6.441**	**< 0.0001**	**3.960**	**8.227**	**0.360**	
	AgeGroup5	**2.147**	**1.203**	**163.618**	**1.785**	**0.0762**	**-0.754**	**4.745**	**0.138**	
	Sex (Female)	**-2.208**	**0.336**	**1039.583**	**-6.563**	**< 0.0001**	**-2.868**	**-1.548**	**0.199**	
Lactation Duration ~ SNP + Age										0.223
SERT	**(Intercept)**	**15.461**	**0.715**	**107.050**	**21.619**	**< 0.0001**	**14.046**	**16.884**	**0.902**	
	*SERT*_12472	0.480	0.324	98.396	1.482	0.141	-0.167	1.118	**0.148**	
	**SERT*_15549	-0.244	0.174	105.123	-1.406	0.163	-0.586	0.106	0.136	0.00238
	SERT_15636	-0.143	0.239	97.675	-0.6	0.550	-0.615	0.332	0.061	
	AgeGroup2	**1.904**	**0.262**	**118.231**	**7.258**	**< 0.0001**	**1.350**	**2.429**	**0.555**	
	AgeGroup3	**2.681**	**0.292**	**174.740**	**9.188**	**< 0.0001**	**2.101**	**3.256**	**0.571**	
	AgeGroup4	**1.877**	**0.404**	**401.346**	**4.643**	**< 0.0001**	**1.082**	**2.671**	**0.226**	
	AgeGroup5	**2.130**	**0.589**	**333.193**	**3.617**	**< 0.0001**	**0.973**	**3.289**	**0.194**	

Bolded values indicate statistical significance ($P < 0.05$). Italicized values are marginally non-significant ($P < 0.1$).

* indicates alternative loci that was linked with SERT_5987 that approached significant effect for trait.

C.2 Additional figures

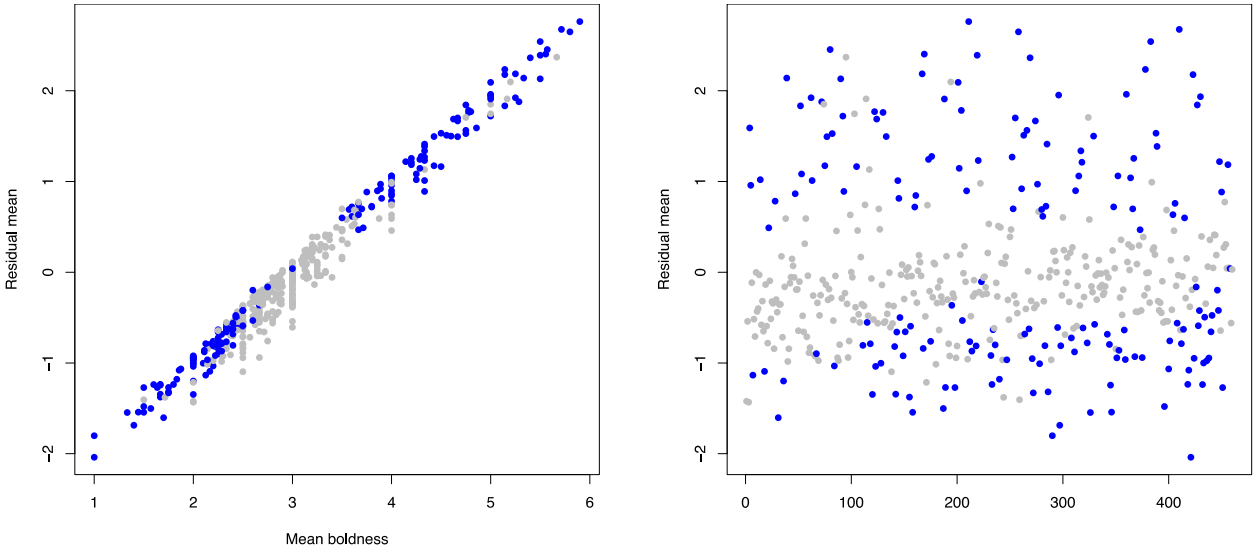

Figure C.1: Plots of residuals to ensure selection of females was appropriate for study objectives. A GLM was performed correcting for age and habitat on boldness using the whole dataset, and subsequently estimated the mean residual for each female and plotted that against the mean raw boldness score (left), confirming selection of females was appropriate. Blue dots represent seals included in the current study and grey dots of females not included. Also plotted are the mean residual values (right). There are instances where females falling into the 'shyest' and 'boldest' regions were not included in the study; however, genetic samples were not available for all females for which boldness scores were obtained.

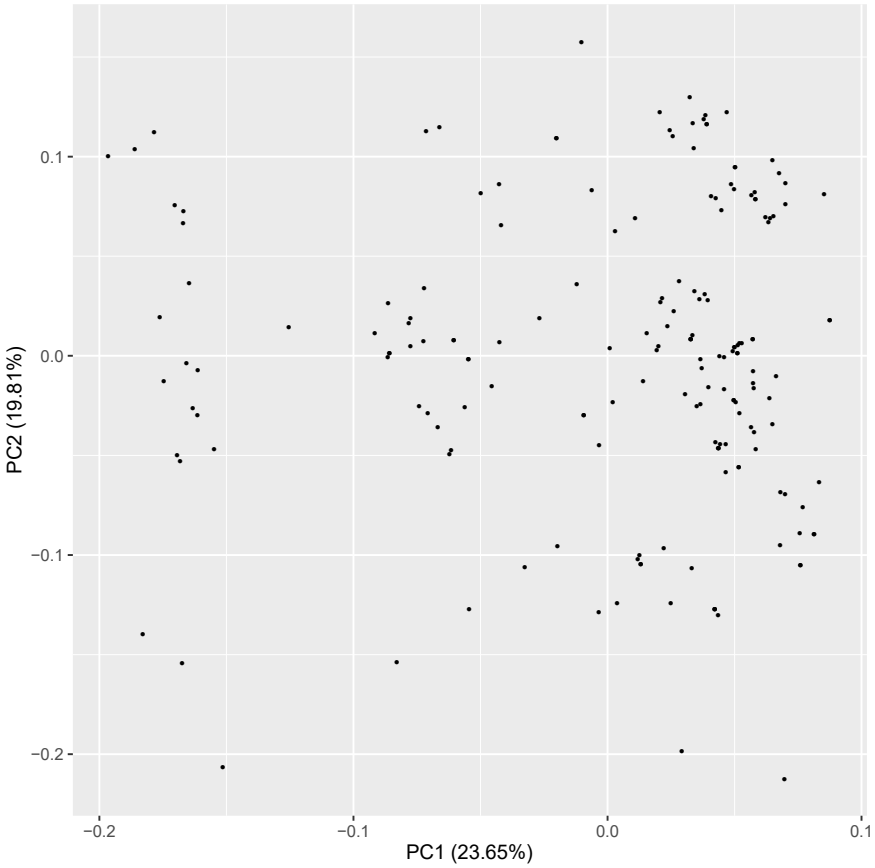

Figure C.2: Principal component analysis (PCA) plot indicating no significant population structure within the Sable Island population of female grey seals of our study. The PCA analysis was completed with missing data imputed on a small number of loci (N = 7) using the R package 'missMDA' (Josse and Husson (2016) missMDA: a package for handling missing values in multivariate data analysis. Journal of Statistical Software. http://dx.doi.org/10.18637/jss.v070.i01) and the PCA was completed using the 'prcomp' function. The imputation of missing data on this small dataset will potentially result in the appearance of clusters. In addition, these loci are distributed on three different genes, and have non-statistically significant levels of linkage. In datasets with no population structure, the appearance of linkage will result in strong clustering of individuals (e.g., Figure 2a in Trevoy et al. (2019) - https://dx.doi.org/10.1002%2Fece3.4803, and Figure 3 in Lumley et al. (2020) - https://doi.org/10.1002/ece3.5950).

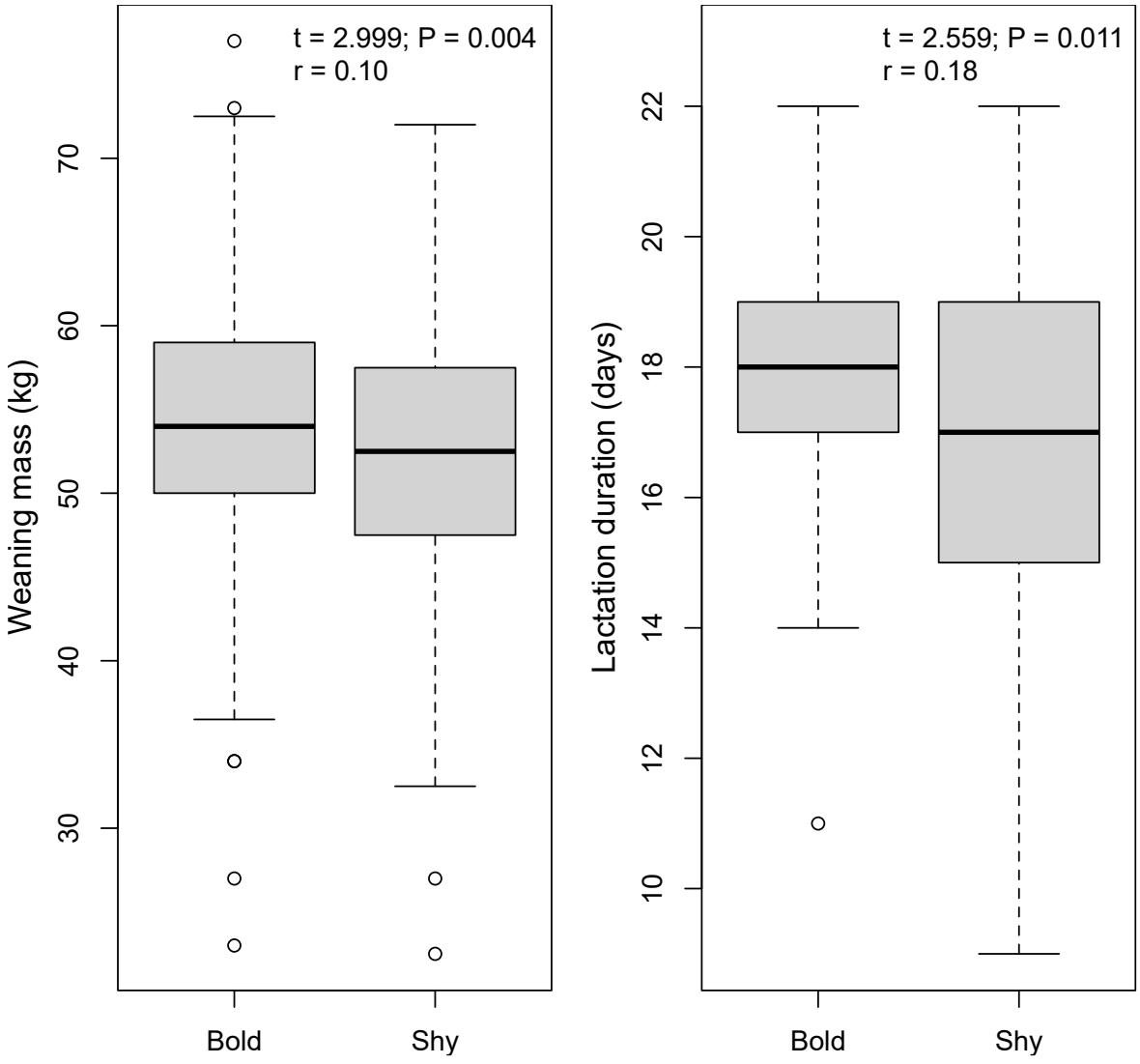

Figure C.3: Boxplot showing the relationships between boldness and two measures of maternal performance, pup weaning mass (PWM) and lactation duration. Horizontal line within the box represents the median values for maternal performance traits measured. In the weaning mass panel, the median value of PWM for bold females was 54 kg (n = 606 observations; range is 22.5-77 kg) and that of shy females was 53 kg (n = 353 observations; range is 27-72 kg). In the lactation duration panel, the median was 18 days (n = 123 observations; range is 9-22 days) for bold females and 17.5 days (n = 98 observations; range is 9-22 days) for shy females.

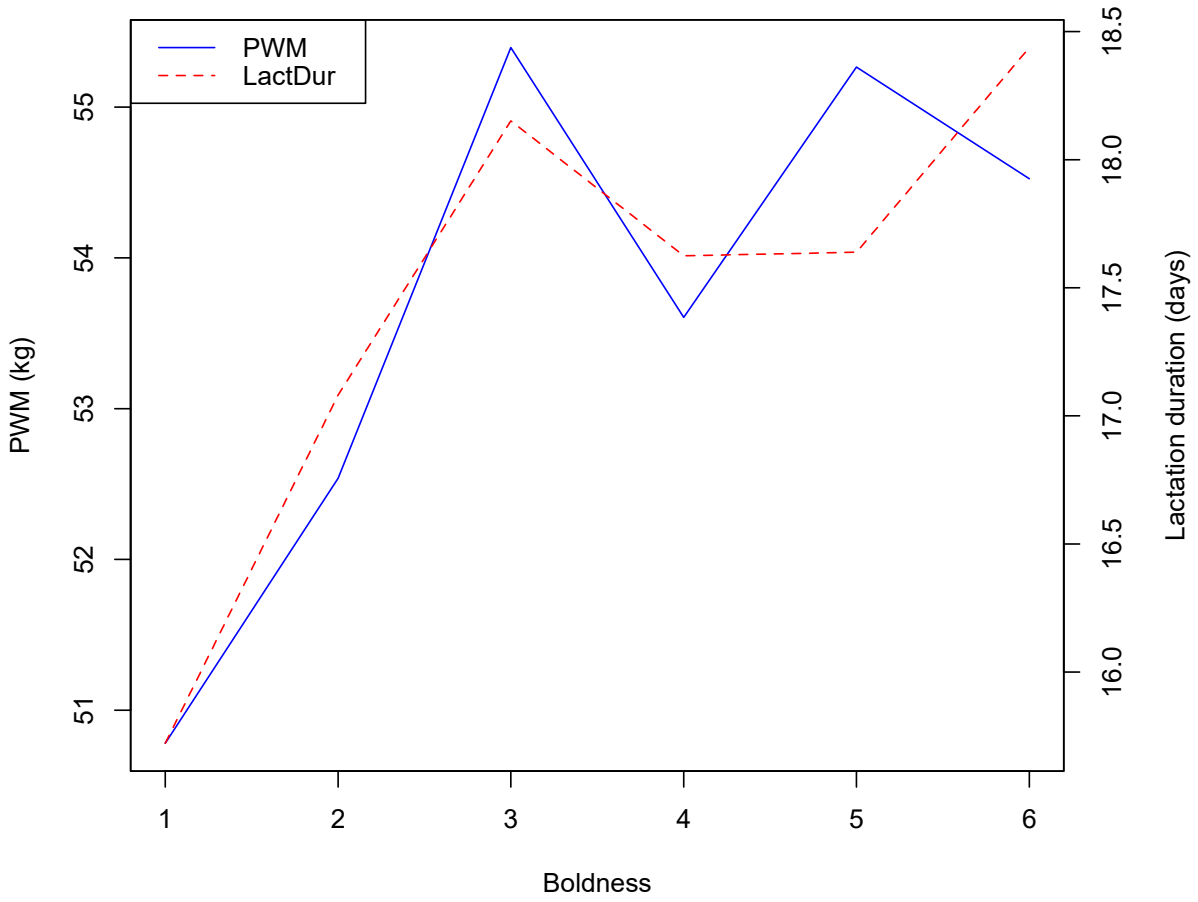

Figure C.4: The relationships between boldness and two measures of maternal performance, pup weaning mass (PWM) and lactation duration, in a subset of Sable Island grey seal females (n = 188) having either extreme shy (scores 1 and 2) or bold (scores 4-6) phenotypes, with repeated measures collected of boldness collected over an 11-year period. A boldness score of 3 (intermediate, no boldness) could be a reflection of shy or bold females in certain years of data collection.

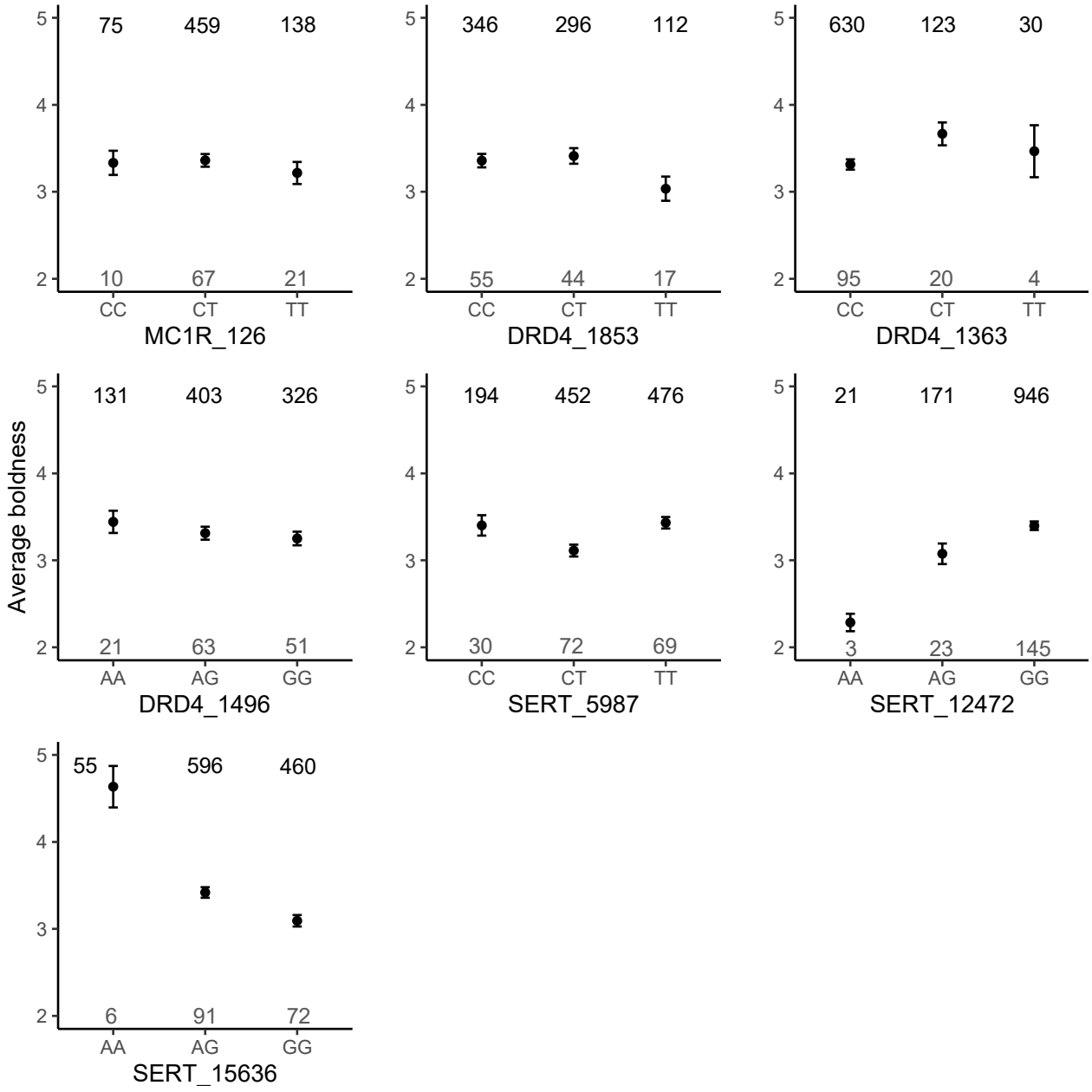

Figure C.5: Boldness scores for female grey seals in relation to SNP genotypes for seven unlinked loci identified across three candidate genes (*MC1R*, *DRD4*, and *SERT*). The number of females and repeated observations used for analyses is provided above each genotype and the genotype mean value, respectively. Error bars represent standard error of the genotype mean. The corresponding test statistics are provided in Table 4.3.

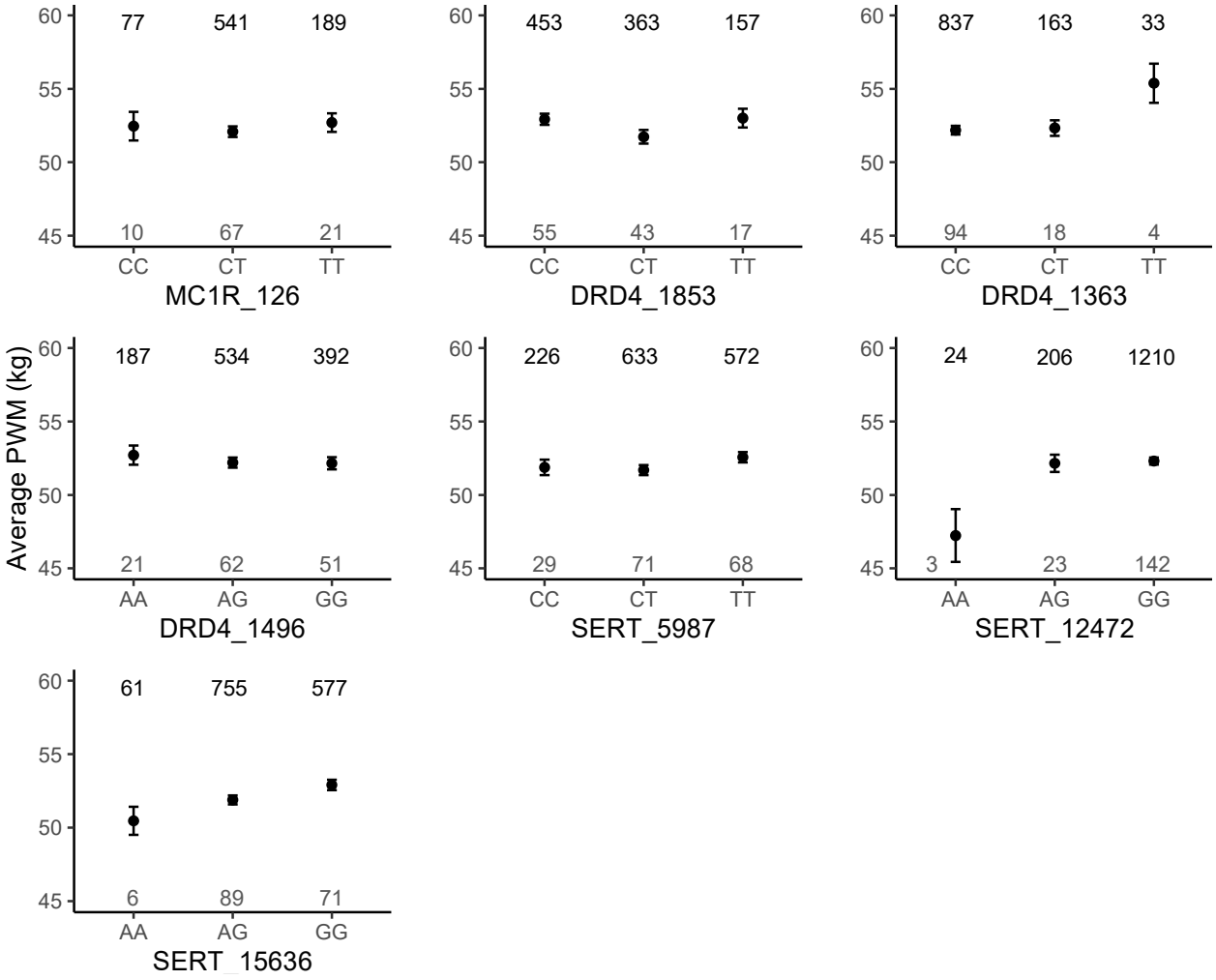

Figure C.6: Pup weaning mass (PWM) values, a reproductive performance parameter for female grey seals, in relation to SNP genotypes for seven unlinked loci identified across three candidate genes (*MC1R*, *DRD4*, and *SERT*). The number of females and repeated observations used for analyses is provided above each genotype and the genotype mean value, respectively. Error bars represent standard error of the genotype mean. The corresponding test statistics are provided in Table 4.3.

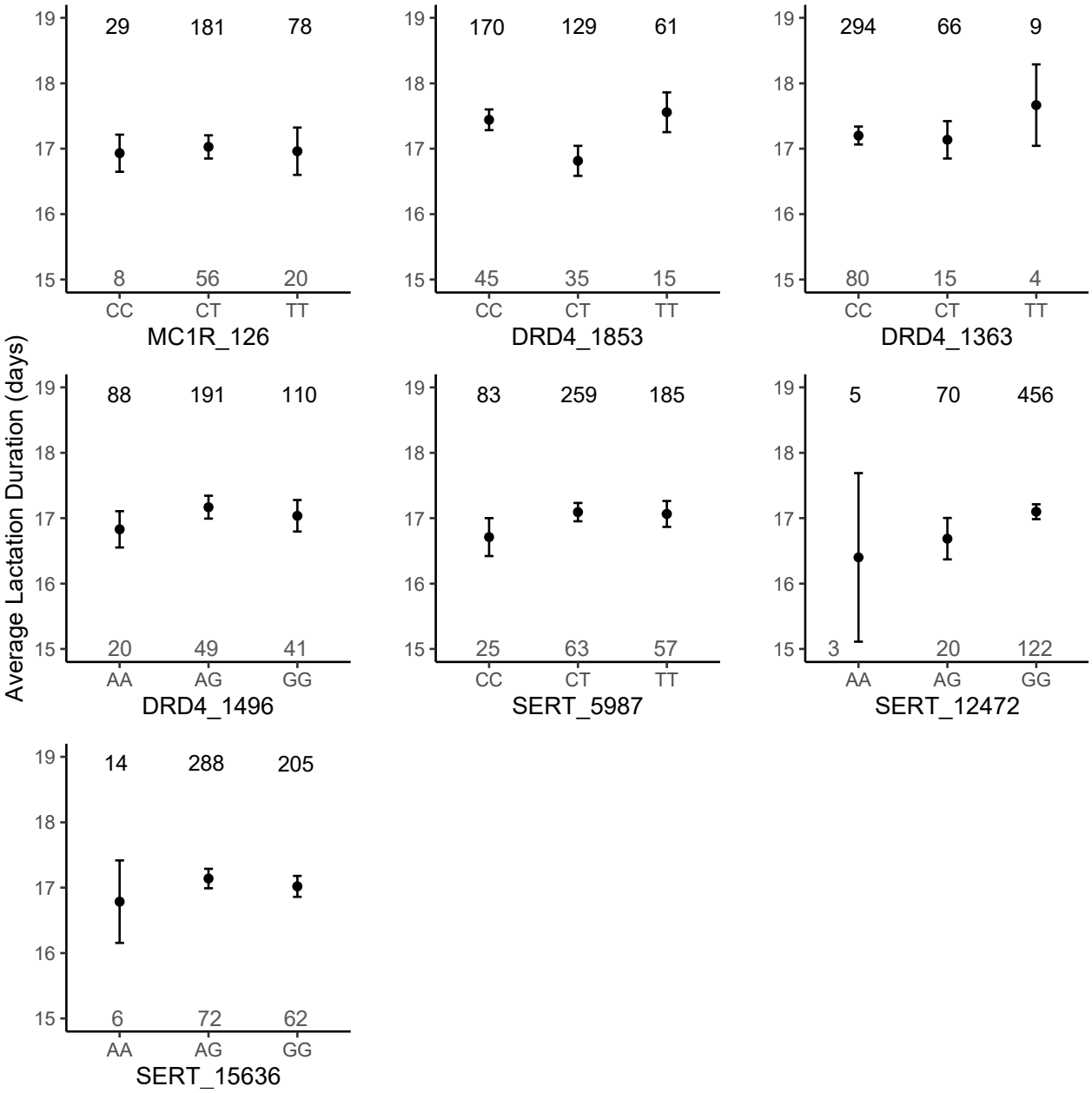

Figure C.7: Lactation duration of female grey seals in relation to SNP genotypes for seven unlinked loci identified across three candidate genes (*MC1R*, *DRD4*, and *SERT*). The number of females and repeated observations used for analyses is provided above each genotype and the genotype mean value, respectively. Error bars represent standard error of the genotype mean. The corresponding test statistics are provided in Table 4.3.

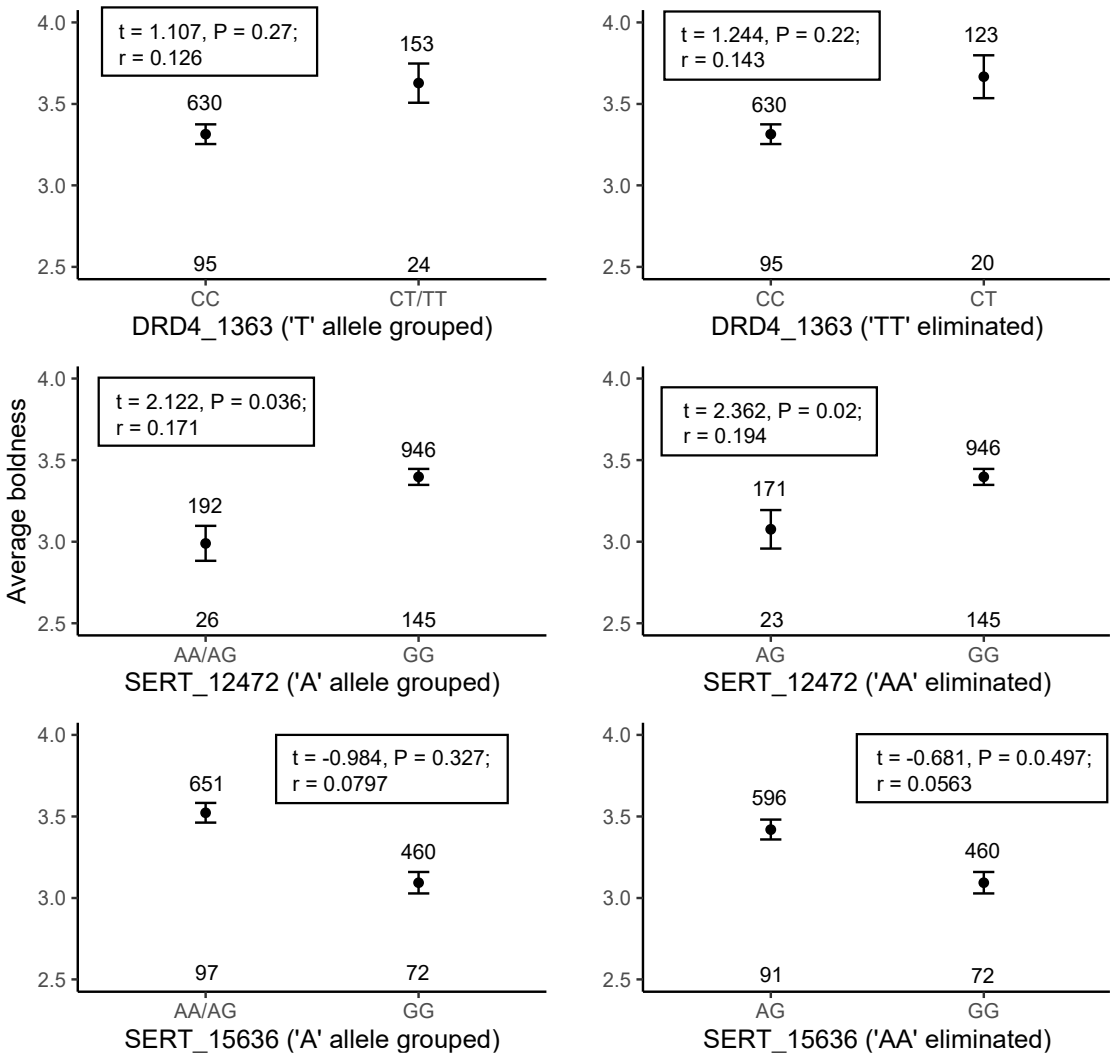

Figure C.8: Boldness scores for female grey seals in relation to SNP genotypes for three loci whose minor allele homozygote genotypes were rare in the population. Grouping the minor allele (allele grouped) and eliminating the rare genotype from analyses (genotype eliminated) was done to ensure that rare genotypes were not disproportionately influencing the relationship between genotype and boldness. The number of females and repeated observations used for analyses is provided above each genotype and the genotype mean value, respectively. Error bars represent standard error of the genotype mean.

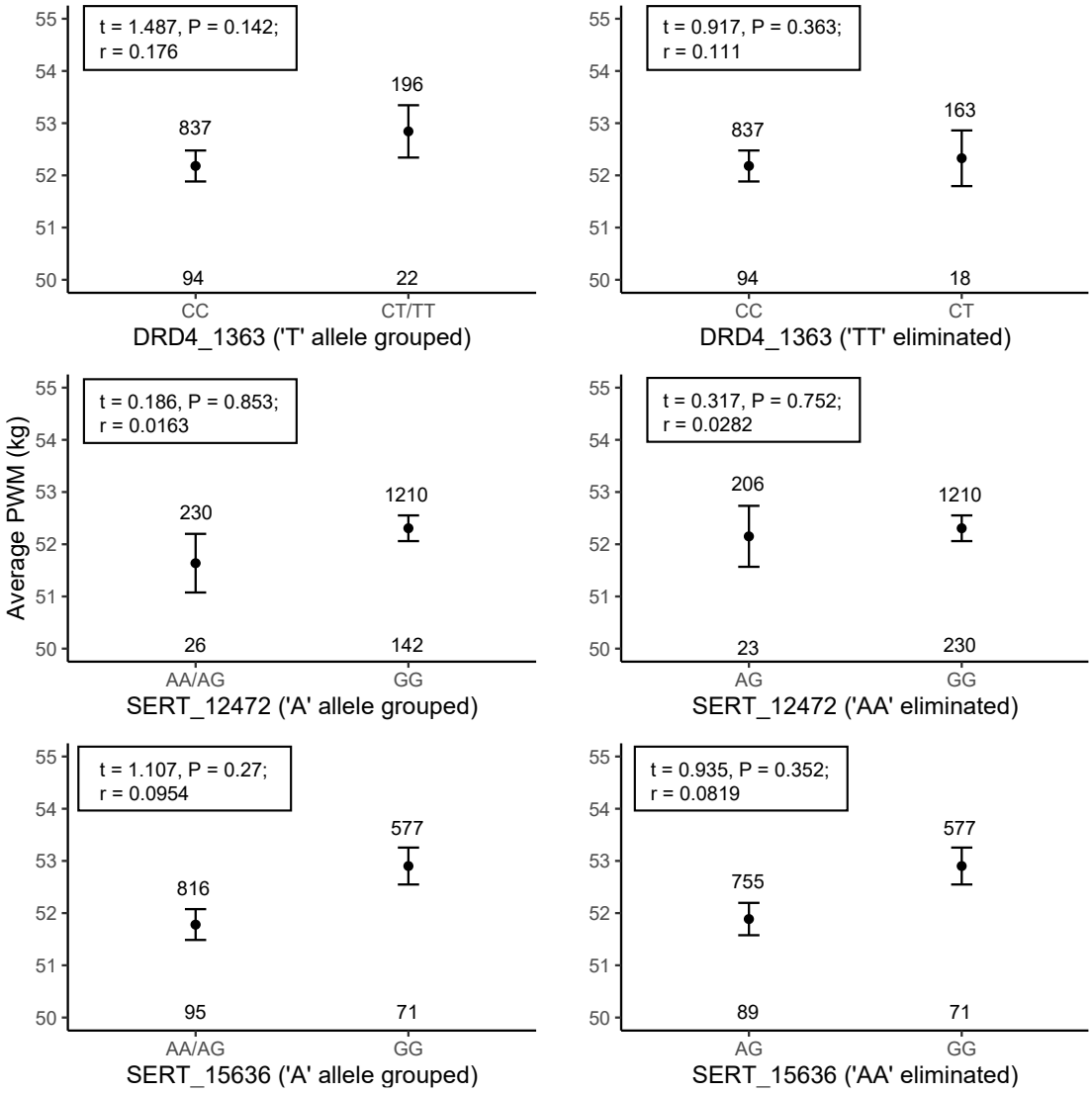

Figure C.9: Average pup weaning mass (PWM) scores for female grey seals in relation to SNP genotypes for three loci whose minor allele homozygote genotypes were rare in the population. Grouping the minor allele (allele grouped) and eliminating the rare genotype from analyses (genotype eliminated) was done to ensure that rare genotypes were not disproportionately influencing the relationship between genotype and PWM. The number of females and repeated observations used for analyses is provided above each genotype and the genotype mean value, respectively. Error bars represent standard error of the genotype mean.

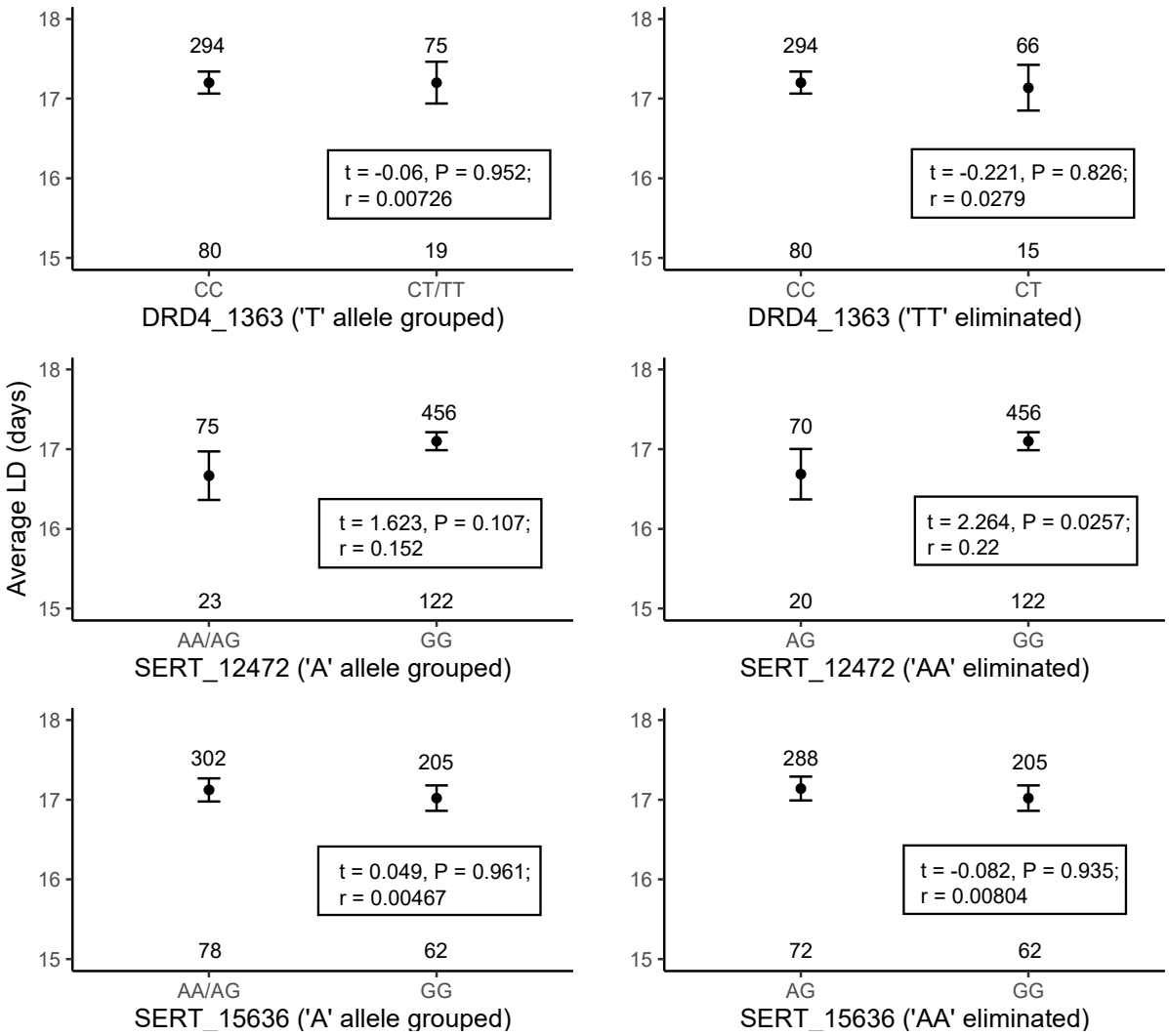

Figure C.10: Average lactation duration (LD) scores for female grey seals in relation to SNP genotypes for three loci whose minor allele homozygote genotypes were rare in the population. Grouping the minor allele (allele grouped) and eliminating the rare genotype from analyses (genotype eliminated) was done to ensure that rare genotypes were not disproportionately influencing the relationship between genotype and LD. The number of females and repeated observations used for analyses is provided above each genotype and the genotype mean value, respectively. Error bars represent standard error of the genotype mean.

Chapter 5: Heritability and association analyses of reproductive performance in wild grey seals

5.1 Abstract

Individual variation provides the material upon which selection acts, and longitudinal studies spanning multiple generations with phenotypic records maintained for hundreds to thousands of individuals have proven invaluable in shedding light on such variation, contributing to our understanding of population dynamics. For such studies with archived tissue banks, there is now potential to explore the genetic basis of trait variation by taking advantage of advances in high-throughput sequencing technologies, enabling identification of genome-wide genetic markers in virtually any species. Here, we used phenotypic records and genetic samples from a multi-decadal (1983-2020) study on a long-lived marine predator, the grey seal (*Halichoerus grypus*), to perform quantitative genetic and genome-wide association analyses on eight maternal traits, representing morphological, life-history, and behavioural phenotypes. Using restriction site associated DNA sequencing, we obtained genotypic data for over 450 female grey seals and determined that six of the eight maternal traits had significant narrow-sense heritability values (h^2) ranging from 0.08-0.38, suggesting existence of adaptive potential in these traits. Our genome-wide association analyses did not reveal any loci that were significantly associated with the traits examined following correction for multiple testing, and thus provides some support for a polygenic architecture underlying the traits examined. Our analyses provide insight into the evolutionary dynamics and adaptive potential of natural populations. These investigations are especially relevant as biodiversity faces continuing cumulative effects of anthropogenic stressors and changing environmental conditions.

5.2 Introduction

Recognizing the existence of consistent individual differences in fitness-related traits within and among populations has an extensive history in evolutionary biology. While much has been learned about individual variation since Darwin's theory of evolution (Darwin 1859), the mechanisms underlying the source and maintenance of variation still remains uncertain.

Following a period where much variation was often regarded as statistical noise around an adaptive mean (Bennett 1987; Wilson 1998), there has been a resurgence of studies with a focus on describing among-individual trait variation within natural populations (Hayes and Jenkins 1997). This resurgence, concurrent with methodological advances in molecular sequencing and statistical methods for genetic analyses, has made it possible to investigate the genetic basis underlying quantitative trait variation in nearly any species. Understanding the genetic basis of complex traits not only provides insight into evolutionary dynamics, such as the source of variation, its transmission across generations, and its maintenance under selection, but also into the evolutionary potential of traits (Gienapp 2020). Having such understanding may permit better inference of the adaptive capacity of individuals within populations (Stapley et al. 2010; Santure and Garant 2018). This is especially relevant as biodiversity faces continuing cumulative effects of anthropogenic stressors and changing environmental conditions.

Next-generation sequencing (NGS) strategies, characterized by widely accessible high-throughput sequencing technologies and decreasing genotyping costs, have permitted the production of high-density panels of genetic markers, typically single nucleotide polymorphisms (SNPs), in 'non-model' organisms (Mackay et al. 2009). These marker panels, often containing thousands of SNPs, make it feasible to interrogate the quantitative and molecular genetic basis of traits in free-living populations. For example, estimating heritability of quantitative traits was previously limited to populations in which relatedness was known via pedigree information or through experimental crosses (Gienapp et al. 2017a). However, NGS data can now readily be used in quantitative genetic analyses (Stapley et al. 2010), expanding our understanding of the adaptive potential in more natural systems than ever before. While using genetic markers to assess relatedness is not novel (Garant and Kruuk 2005), estimates from large-scale genomic datasets offer increased precision and reliability compared to those calculated using smaller marker datasets (i.e. microsatellites) (Gienapp 2020). This is due in part to the determination of realized genomic relatedness between individuals of a population (Gienapp et al. 2017a). Though heritability makes it possible to infer the adaptive potential in a particular population and environment, it cannot provide insight into the networks of genes underlying trait variation. Therefore, integrating quantitative genetics with molecular genetic analyses, such as genome-wide association analyses, may aid in determining the underlying genetic architecture (e.g., number of loci, genomic distribution of loci, and magnitude of loci effect) of complex traits

within and among wild populations, and notably of species with little to no *a priori* genomic information (Santure and Garant 2018).

Despite these promising applications in the wild, their use has thus far been limited to a handful of natural systems (Slate et. al. 2010; Gienapp et al. 2017a; Bubac et al. 2020). One limitation is obtaining sample sizes of phenotypes and genotypes to provide adequate statistical power, already difficult in sampling free-ranging individuals, especially in small populations (Santure and Garant 2018). However, longitudinal field studies that span several decades and multiple generations, many of which contain extensive phenotypic datasets, have already proven valuable in shedding light on the ecological and genetic processes affecting fitness (Clutton-Brock and Sheldon 2010a). In some longitudinal studies, individual-based records are maintained for various traits across the lifetime of hundreds to thousands of individuals (Clutton-Brock and Sheldon 2010b; Stapley et al. 2010); thus, providing the sample sizes needed for quantitative and molecular genetic investigations. Longitudinal studies on natural systems, such as the great tit (*Parus major*), Soay sheep (*Ovis aries*), and bighorn sheep (*Ovis canadensis*), have contributed to our understanding of the evolutionary dynamics and adaptive potential of morphological (Bérénos et al. 2015; Miller et al. 2018), life-history (Santure et al. 2013), and behavioural traits (Kim et al. 2018).

A multidecadal field study on the grey seal (*Halichoerus grypus*) provides an opportunity to examine the genetic basis of quantitative traits in a free-ranging marine system. Grey seals are long-lived animals, with life spans of 30-40 years (Bowen et al. 2006), and are philopatric, colonial breeders wherein individuals haul out on their natal grounds each year to give birth and to mate (Pomeroy et al. 1994; Bowen et al. 2015). Like many other marine mammal species, grey seals were severely exploited for centuries, resulting in the reduction of colonies to exceptionally low numbers or local extinctions (den Heyer et al. 2021). For example, numbers of grey seals in the Northwest Atlantic were estimated to be as low as a few thousand individuals in 1960 (Lesage and Hammill 2001). Following federal protection in the United States and reduced hunting in Canada (Wood et al. 2020), grey seals in the Northwest Atlantic area have since rebounded, now numbering around 424,000 individuals (Hammill et al. 2017). As expected, a genetic signature of a bottleneck event is evident in contemporary samples of Northwest Atlantic grey seals, with large variance and a shift in allele frequencies observed over time indicating

possible selection for certain traits (Cammen et al. 2018b). The species' polygynous mating system and low effective population size also likely contributes to observed genomic patterns.

Female grey seals provide all parental care, giving birth to a single pup on a near annual basis beginning at the age of 4-6 years and are capable of reproducing into their late 30s and early 40s (Bowen et al. 2006). Maternal care in this species is characterized by a female's capacity to deliver enough milk energy to her offspring during a brief, yet intensive, lactation period that lasts 16 to 18 days (Boness and James 1979; Bowen et al. 1992; Iverson et al. 1993). Offspring mass gained, largely in the form of blubber, must sustain the pup through a post-weaning fast that lasts approximately three weeks until the pup reaches foraging independence (Noren et al. 2008). The pup's condition at weaning, therefore, influences early survival, such that larger and fatter pups have an increased probability of surviving to at least age one (Hall et al. 2002; Bowen et al. 2015). Pup weaning mass (PWM) in this species represents one of the best predictors of maternal performance, having an influence on a female's lifetime reproductive success (Mellish et al. 1999). As female grey seals exhibit a capital breeding strategy, where individuals fast for the duration of time spent hauled out pupping and mating, a female's condition and energy stores at parturition affect her maternal performance (Iverson et al. 1993). While these accumulated energy reserves prior to giving birth are informative for maternal performance, additional factors such as capacity to care for offspring, both physiologically and behaviourally, also influence performance (Lang et al. 2009; Bubac et al. 2018; Badger et al. 2020). Maternal performance variation in this species has been well-studied, and individual differences have been found to account for an appreciable proportion of total variance observed across multiple traits. In the largest breeding colony of grey seals, repeatability estimates for birth date ($R = 0.66$; Bowen et al. 2020) and for PWM were high ($R = 0.48$), as were traits that determine total milk energy intake and therefore PWM: milk composition ($R = 0.38$-0.5), daily milk output ($R = 0.46$), and lactation duration ($R = 0.42$-0.57; Lang et al. 2009). In addition, a relationship between a female's shy-bold phenotype and PWM was detected, again exhibiting strong inherent differences between individuals in boldness scores ($R = 0.61$) (Bubac et al. 2018). Selection for consistent individual differences in the grey seal, therefore, is strongly supported.

While repeatability estimates are believed to set the upper bounds to heritability (the proportion of phenotypic variance explained by additive genetic variation) by including both

environmental and genetic sources of trait variation within a population (Falconer and Mackay 1996), this is not always the case and instances exist where repeatability estimates do not accurately reflect those of narrow-sense heritability (Dohm 2002). High repeatability estimates as described by Lang et al. (2009), Bubac et al. (2018), and Bowen et al. (2020) in the grey seal suggest that testing for trait heritability and further elucidating the molecular genetic basis of various maternal performance traits may be possible. In this study, we used both a 'top-down' and 'bottom-up' approach to explore the genetic basis of maternal performance in the wild population of grey seals on Sable Island National Park Reserve of Nova Scotia, Canada. A subset of individuals belonging to this population are especially well-studied, owing to a long-term life-history monitoring program spanning several decades (Bowen et al. 2006). The objectives of our study were threefold. First, we used a restriction site-associated DNA sequencing (RADseq) technique to develop a panel of polymorphic SNP markers located throughout the grey seal genome. Second, using this genomic marker data, we estimated additive genetic variance and determined heritability for one morphological trait (maternal mass), six life-history traits (female length at first parturition, age at first parturition, parturition date, pup birth mass, PWM, lactation duration), and one behavioural trait (boldness) using animal models (Henderson 1984). Traditionally used in animal breeding, the animal model is a linear mixed-effects model that, in addition to other factors, takes into account genetic relatedness between individuals, and is used to estimate additive genetic variance (Kruuk 2004; Wilson et al. 2010), and thus heritability of traits. Finally, we performed genome-wide association analyses using SNP data to determine whether specific loci are associated with the measured traits.

5.3 Methods

5.3.1 Study site

This study was conducted on Sable Island National Park Reserve (hereafter Sable Island) located approximately 300 km due east of Halifax, Nova Scotia (Canada) (43°55'49'' N, 60°00'67'' W). Sable Island is roughly 40 km long and 1.5 km wide at its widest point and is characterized by various microhabitat features including sand inland, dune, and beach areas as well as vegetated dune habitat. The island supports the largest breeding colony of grey seals (den Heyer et al. 2021), with an estimated 370,000 individuals that haul out annually from December to early February to give birth and to mate (Hammill et al. 2017). This colony of grey seals has

been studied annually by Canada's Department of Fisheries and Oceans (DFO) since the 1960s, with more intensive research effort beginning in 1983. Thus, this investigation represents one of the most extensive longitudinal studies on a marine mammal species with which individual-based records have been maintained. A subset of individuals in the population were permanently marked as weaned pups with a unique 3- or 4-character hot-iron brand applied to the seal's lower back region. These branded individuals are of known age and can be visually identified and followed through their lifetime. At the time of branding, a small tissue sample was collected from the pup's hind flipper and later archived in a tissue bank (4384 samples in total). Each year, Sable Island is systematically searched by a team of 6-10 researchers using all-terrain vehicles on a nearly weekly basis during the breeding season to identify and record the location of all branded seals (Bowen et al. 2006). Research efforts are focused on assessing population recruitment, demographics, and locating and monitoring the reproductive status of branded females. Live-capture and handling techniques complied with the Canadian Council on Animal Care and were performed under permits issued by DFO Canada. All animal use protocols were reviewed and approved by the DFO Canada's and the University of Alberta's Animal Care Committees.

5.3.2 Maternal trait data collection

Given logistical constraints and resource limitations, a subset of branded females, termed life-history females, were randomly selected to collect long-term field data annually. Life-history females in our study represented the following cohorts: 1970, 1973, 1974, 1985-1987, 1989, and 1998-2002 (Table D.1). Once located in the breeding colony, we recorded each life-history female's reproductive status: not pregnant, pregnant, or if postpartum, her pup's developmental stage (see Bowen et al. 2003) (Table D.2). The weekly, island-wide censuses as described above allowed for determination of a female's age at first parturition. If pregnant or with pup, females were monitored from a distance, to reduce disturbance to her and her offspring, daily. Such intensive monitoring permitted accurate determination of birth date according to the pup's developmental stage. Developmental stage one, lasting approximately one to two days, is characterized by the pup having loose skin folds and yellowish pelage that is still wet from birth fluids (Bowen et al. 2003). The birth date of pups beyond this stage (i.e. stage two and older) could not accurately be determined.

On day-three postpartum or later, three researchers approached the female and her pup and applied a uniquely numbered tag to the webbing of the pup's hind flipper to allow for identification once weaned. At the time of tagging, a female's shy-bold phenotype was recorded as determined by her response to a potentially risky situation (i.e. researcher approach) and in defense of her offspring (see Bubac et al. 2018 for details). A behavioural score of one represented very shy females and of six very bold individuals (Table D.3). Interactions typically lasted under one minute and were performed a few days after birth to allow for adequate bonding to occur between mother and offspring. Also, at day-three postpartum, some females were captured to obtain measurements of weight (to the nearest 0.5 kg) and for primiparous females only, standard dorsal body length (snout to base of tail measured to the nearest cm). At this time, pups were also weighed to the nearest 0.5 kg to assess individual differences in birth mass and early reproductive performance. Females that were captured, or who's pup was weighed at this time, did not receive a boldness score in that breeding season.

Female grey seals wean their offspring abruptly, returning to the ocean to resume foraging; thus, daily monitoring permitted verification of weaning date. For females with which both birth and weaning date were known, lactation duration was calculated. Weaned pups were identified by their hind flipper tag, sexed, and weighed to the nearest 0.5 kg. Although data on birth date, pup birth mass, maternal mass, lactation duration, and PWM were collected beginning in 1991, measures of standard dorsal body length and boldness were not collected until 1997 and 2008, respectively. Data collection on a female's age at primiparity began in 1973. Scoring boldness ceased in the 2018-2019 breeding season, while values for all other traits were available through the 2019-2020 season. Prior to analyses, phenotypic data was visually examined to detect and remove outlying measurements that were improbable or environmentally influenced (i.e. storm-induced), and thus not a direct reflection of a female's performance. For instance, strong storm surge waves may cause premature mom-pup separation, and in some cases, result in vastly decreased lactation duration periods (e.g., 1-4 days).

5.3.3 ddRAD library preparation and post-processing

Whole genomic DNA was extracted from 485 tissue samples using Qiagen DNeasy Blood and Tissue extraction kits according to the manufacturer's protocol (Qiagen, Valencia, California, USA). DNA extracts were quantified using the Qubit Fluorometer (Life

Technologies) and subsequently standardized to 20 ng/μl. We prepared double-digest restriction site-associated DNA (ddRAD) sequencing libraries (Peterson et al. 2012) according to MacDonald et al. (2020) with few modifications. Briefly, individual genomic DNA was digested using the restriction enzymes SbfI and EcoRI and subsequently ligated with adaptors. One of 16 individually 8-bp indexed P1 adaptors and a single P2 adaptor were ligated to the SbfI and EcoRI sites, respectively. Ligated samples were then pooled into groups of 16 individuals, each containing unique indexes, and subsequently cleaned using a QIAquick PCR Purification Kit (Qiagen) to remove excess adaptors and buffers. Libraries were PCR amplified, adding an index to facilitate pooling and the sequence required for flow cell annealing. A total of 35 libraries were sequenced in three runs of a Nextseq 500 (Illumina, San Diego, CA, USA), with 75-bp single-end read sequencing. Details of methodology used for library preparation can be found in Appendix D.1. Libraries were prepared and sequenced at the University of Alberta's Molecular Biology Service Unit.

Quality of RADseq data was initially checked using FastQC (Andrews 2010). Sequences were then processed using the software STACKS 2.0b (Catchen et al. 2011, 2013) to assemble loci *de novo* as a reference genome was not available at the time of the assay. Following quality filtering and demultiplexing with STACKS's *process_radtags* program, reads were trimmed to 67 bp with the removal of the 8-bp index sequence following Miller et al. (2021). We then used the *de novo map* pipeline of STACKS, which involves core components (*ustacks*, *cstacks*, and *sstacks*), wherein three main parameters (*m*, *M*, and *n*) affect how loci are built *de novo*. The *m* and *M* parameters in *ustacks* determine the minimum number of reads required to form a stack (putative allele), and the number of mismatches between stacks that are allowed to occur within population samples, respectively. Parameter *n* in *cstacks* defines how many mismatches are permitted between loci to build a catalog across population samples. Parameter values chosen while navigating through the *de novo* pipeline are study-specific (e.g., species and experimental design), and can greatly influence downstream analyses and results (Paris et al. 2017; Díaz-Arce and Rodríguez-Ezpeleta 2019). As such, we followed recommendations as provided by Paris et al. (2017), iterating values of *m* (m1-m6), *M* (M0-M6), and *n* (n0-n6) while fixing the other two parameters to default values (m = 3, M = 2, n = 0), in an effort to optimize our RADseq data. In addition, we followed the *r80* loci rule (present across 80% of individuals) throughout iterative runs to increase the likelihood that loci constructed were not paralogs or from repetitive sequence

(Paris et al. 2017). Following iterative runs of parameter values in a subset of 30 grey seals that were selected randomly from across cohorts, sequencing runs, and with varying sequence coverage per individual, we extracted and plotted assembly metrics (e.g., number of assembled loci, polymorphic loci, and SNPs) (Figure D.1). Optimal values for *m*, *M*, and *n* were chosen based upon visual examination of the plots (see Paris et al. 2017). Upon selection of values that maximized detection of polymorphic loci and SNPs, the parameter combination was applied to the full dataset (N = 476 individuals) following the removal of nine samples due to low coverage.

After calling SNPs, we used VCFTOOLS 0.1.14 (Danecek et al. 2011) to filter the dataset by genotype quality (minimum quality score of 30) and a minimum minor allele frequency (MAF) threshold of > 0.01. Using PLINK 1.9 (Purcell et al. 2007), we further removed individuals and loci with > 10% missing data. PLINK was also used to determine which loci were in statistical linkage disequilibrium (LD) using the *r*2 method and comparing all possible pairwise combinations. Results from LD analysis were corrected for multiple testing in R using the *p.adjust* command and false discovery rate (FDR) correction method. We corrected for LD by using the 'leave-one-out' method for linked SNPs. Following the removal of individuals and loci with too much missing data as well as correcting for linked loci, we tested for SNP deviation from Hardy-Weinberg equilibrium (HWE; $\alpha = 0.05$). We flagged SNPs that deviated from HWE but retained these loci for analyses. While deviating loci could indicate sequencing error, these loci could potentially be under selection, and would, therefore, be expected to depart from HWE conditions.

5.3.4 Base model selection

While an additive genetic effect may explain phenotypic variation in the traits we are interested in, other factors are known to affect variation in the traits under investigation (e.g., Lang et al. 2009, Bowen et al. 2015, Bubac et al. 2018). Therefore, prior to performing SNP-based analyses, we determined the minimum adequate model containing non-genetic effects for each of the two single measure traits (female length at first parturition and age at first parturition) and the six repeated measures traits (maternal mass, birth date, pup birth mass, PWM, lactation duration, and boldness). Models were selected according to Akaike information criterion (AIC) values (i.e., lowest AIC, smallest ΔAIC, and highest AIC weights) (Burnham and Anderson

2002), while taking into account missing data for putative fixed factors. These models identified the random (individual ID, year, cohort) and fixed (e.g., maternal age, parity, pup sex, and birth site habitat selection) factors that should be accounted for in the base models used for quantitative genetic and association analyses. Only significant terms were retained in final models. In this population, birth and wean date are highly correlated (Spearman's correlation $r = 0.92$, $P < 0.0001$), and as more records were available for wean date than of birth date in our dataset, we used weaning date as a proxy for birth date. Weaning date was determined by the number of days following the start of the pupping/breeding season (November 30th). Maternal age and parity are similarly highly correlated ($r = 0.95$, $P < 0.0001$); therefore, age or parity was included as a fixed factor where appropriate. We were unable to account for possible maternal effects on adult female traits (e.g., boldness, maternal mass, length), as mom ID was known for only 85 focal, life-history females in our dataset. Factor inclusion and base model selection are provided in Tables 5.1 and D.4. All methods were run in the RStudio v1.3.1056 environment with R v4.1.0.

5.3.5 Quantitative genetic and genome-wide association analyses

Repeatability estimates for repeated measures (≥ 2 measures) traits were calculated using the R package MCMCglmm v2.32 (Hadfield 2010) and from script adapted from Roche et al. (2016). For estimating additive genetic variance and GWAS, we used the R packages *GenABEL* v1.8-0 (Karssen et al. 2016) or *RepeatABEL* v1.1 (Rönnegård et al. 2016) depending on if the trait being examined was single measure or repeated measures, respectively. From the SNP data, a genome-wide relationship matrix (GRM) containing all pairwise relatedness estimates was first generated using the 'ibs' function in *GenABEL*. This GRM was added as an additional random factor to the base model to correct for underlying population structure.

The methods used by *GenABEL* and *RepeatABEL* varied slightly for single measure versus repeated measures traits. For single measure traits, we used the 'polygenic_hglm' function, which estimates a polygenic model using a hierarchical generalized linear model (Lee and Nelder 1996; Rönnegård et al. 2010). In *RepeatABEL*, a two-step modeling procedure was used. First, a base linear-mixed effect model was fit without genotype effects. Here, models varied in their structure depending upon the trait, but always included the GRM and individual ID as random factors to account for polygenic effects and repeated measures, respectively. From

these model structures, additive genetic variance components were extracted to estimate trait narrow-sense heritability, calculated as the ratio of additive genetic variation to total phenotypic variation ($h^2 = V_A/V_P$). Marker-based estimates of heritability were used as no pedigree is available in this system to calculate heritability via traditional methods such as parent-offspring regression or an 'animal-model'; however, we note that Perrier et al. (2018) have shown that GRM and pedigree-based methods perform equally well, with a slight underestimation with the pedigree approach. Standard error for each heritability estimate was calculated based on code from Silva et al (2017), which was extended to allow for additional random factors (year and cohort) beyond the GRM and individual ID for some repeated measures traits. To examine the relationship between repeatability and heritability estimates, we used reduced major-axis regression with the R package lmodel2 v.1.7.3 (Legendre 2018).

The second step in *RepeatABEL* used the estimated (co)variance matrix generated in the first model-fitting step to test for associations between individual SNPs and phenotypic values. Specifically, associations were assessed using a linear model and p-values calculated with a Wald statistic. Individual SNP effects from all models were then visualized with Manhattan plots created using ggplot2 v3.3.2 (Wickham 2016). A strict Bonferroni adjustment was used to correct for multiple testing at an alpha level of 0.05. We further calculated the genomic inflation factor (λ) to evaluate whether population structure likely influenced association results. Stratification, if left unaccounted for, could lead to false positive associations (Freedman et al. 2004; François et al. 2016).

To add genomic context to the identified SNPs, we mapped the flanking regions surrounding each SNP identified within STACKS to the newly generated grey seal chromosome level assembly (dnazoo.org; Dudchenko et al. 2017) using bowtie2 with the sensitive flag. Mapped reads were further processed by sorting (SAMtools, Danecek et al. 2021) and converting to bed files (BEDTools, Quinlan and Hall 2010) for generating Manhattan plots.

5.4 Results

5.4.1 SNP parameter testing

After applying quality filters, ddRAD sequencing yielded a total of 650,477,226 reads, with an average of 1,366,549 reads per sample (SD = 494,255.6) among 476 individuals that were sequenced. Following parameter selection recommendations by Paris et al. (2017), the

optimal Stacks parameters empirically determined for our dataset at r80 were $m = 3$ and M and $n = 2$ (Figure D.1). These values provided us with the highest number of polymorphic loci and SNPs while minimizing potential error. After quality control checks and pruning for missing data, MAF, and LD, 1453 SNPs were retained for analyses. Of these markers, 1430 SNP flanking regions aligned once to the grey seal reference genome and 118 aligned more than once.

5.4.2 Quantitative genetic estimates and GWAS

Post-quality filtering and consideration of completeness of genotypic and phenotypic data (i.e. minimal missing data) yielded a sample size of up to 460 females and 4727 repeated observations per trait (Table 5.2). Only lactation duration showed extreme environmentally-driven outliers, and as such, we removed four records of shortened lactation duration values for biological rather than statistical reasons that resulted from premature, storm-related separations.

Repeatability adjusted for fixed factors was moderate to high for boldness (R = 0.58 [CI: 0.53-0.62]), maternal mass at parturition (R = 0.59 [CI: 0.45-0.74]), PWM (R = 0.4 [CI: 0.36-0.44]), and birth date (R = 0.48 [CI: 0.38-0.57]). Levels of repeatability were lower for lactation duration (R = 0.23 [CI: 0.17-0.29]) and pup birth mass (R = 0.04 [CI: 0.0073-0.23]) (Table 5.2; Table D.5). We found significant marker-based heritability estimates that ranged from 0.08 to 0.38 for maternal mass, female length at first birth, wean date, lactation duration, PWM, and boldness (Table 5.2). Pup birth mass and maternal age at first parturition were not significantly heritable, as standard errors of the estimates included 0. The magnitude of heritability estimates was uncorrelated to the number of samples used (r = -0.31, P = 0.45), nor was it significantly related to whether the trait was single or repeat measured (r = 0.51, P = 0.19). Repeatability and heritability were positively correlated (r = 0.92, P = 0.026) among significant, repeat-measured trait estimates (Figure D.2).

Results of our GWAS to search for individual loci underlying behavioural and life-history traits are shown in the Manhattan plots (Figure 5.1). In all cases, genomic inflation (λ) was ~1 (Table 5.2), indicating that the genome-wide relatedness matrix had sufficiently accounted for underlying population structure. Following correction for multiple testing, no loci were significantly associated with the traits examined.

5.5 Discussion

A lag in understanding the genetic basis of complex traits exists in free-living populations. By taking advantage of cost-effective genomic reduced-representation sequencing methods and newer analytical techniques, direct inference of the adaptive potential, as well as of the underlying genetic basis for that potential, can be explored. Here, we integrated genomic techniques and analytical strategies with individual-based phenotypic records from a longitudinal field program to explore the additive genetic variance, heritability, and genetic association of morphological, life-history, and behavioural trait variation in a wild grey seal population. Using genome-wide SNP data, we discovered that maternal traits examined were repeatable and had an 8%-38% heritable component. We further found suggestive evidence that all traits examined likely have a polygenic basis, wherein no locus in our dataset explained an appreciable proportion of trait variation. Our study is the first to report heritability estimates in phocids, and these estimates provide important insight into the capacity for evolutionary response to selection in Sable Island grey seals. Generally, this study shows that traits of a recovering pinniped population are capable of responding to selection pressures, while adding to a growing list of studies exploring the proximate mechanisms of natural trait variation (Bubac et al. 2020).

5.5.1 Quantitative genetic assessment

Across taxonomic groups, average heritability estimates for life-history traits ($h^2 = 0.26$; Mousseau and Roff 1987) and behaviour ($h^2 = 0.24$; Dochtermann et al. 2019) indicate a moderate genetic influence, whilst morphological traits tend to have a higher heritable component ($h^2 = 0.46$; Mousseau and Roff 1987). Though we discovered slightly lower-than-average heritability values for the eight grey seal maternal traits examined, our estimates are highly consistent with those of similar traits reported in other system-specific studies [e.g., body length in fur seals (*Arctocephalus tropicalis;* $h^2 = 0.21$-0.37) (Authier et al. 2011); parturition date in red squirrels (*Tamiasciurus hudsonicus*; ($h^2 = 0.16$) (Réale et al. 2003); and, boldness in wandering albatrosses (*Diomeda exulans*; $h^2 = 0.24$) (Patrick et al. 2013)]. These heritability estimates provide support that genetic variation underlying certain ecologically relevant traits exist in the Sable Island grey seal population, and that adaptive responses are indeed possible for these traits.

Length at first parturition had a moderate heritable component ($h^2 = 0.38$) and was highest of all traits examined. Recent studies on body length in Sable Island grey seals suggest a selective advantage to having a larger skeletal size, such that longer individuals may benefit in their foraging abilities and in predator evasion (Bowen et al. 2015). Furthermore, young females that are longer typically reproduce more and tend to have larger pups than their shorter counterparts, contributing to significant individual differences in lifetime reproductive success (Badger et al. 2021). The beneficial effects of length across generations becomes evident – a female's reproductive fitness increases by producing longer pups that, among female pups that recruit to the population, then in turn become more productive mothers themselves (Badger et al. 2021). Though females that recruit are typically longer at weaning in the population, the effects of length at primiparity are reduced (Bowen et al. 2015). Nonetheless, our study reveals the genetic basis of primiparous length, and provides support for the adaptive capacity of this trait.

Low heritability values for other performance traits suggest that genetic influence may be weak in comparison to other sources of trait variation. While heritability provides a means to understand what the speed of response to selection may be, genetic effects may be masked by other variance components. Female grey seals of Sable Island demonstrate high individual consistency in multiple maternal traits (repeatability = 0.23-0.66; Lang et al. 2009, Bubac et al. 2018; Bowen et al. 2020; this study), metrics that comprise influence by both the environment and genetics to total phenotypic variance. In our study, we found that trait variation was oftentimes explained less by additive genetic variance. Much variability in these traits may therefore be driven by indirect genetic or environmental factors - consistent with the directional selection hypothesis and that complex life-history and behavioural traits are especially influenced by environmental variance (Teplitsky et al. 2009; Schroeder et al. 2012; Liedvogel et al. 2012; Hoffmann et al. 2017). For instance, in our study, heritability was lowest for lactation duration ($h^2 = 0.08$), a trait likely affected by external and individual state variables such as premature mom-pup separations due to storms, excessive harassment from males trying to gain mating access, and the female's condition at birth.

Still yet, heritabilities reported herein may also be reflective of biological patterns inherent to our study system. Grey seals of the Northwest Atlantic underwent a recent bottleneck (Cammen et al. 2018b) and further exhibit a polygynous breeding strategy, lowering the overall effective population size. Specifically, the Sable Island population was reduced to exceptionally

low numbers in the early half of the 1900s (Mansfield and Beck 1977). The population underwent exponential population growth from a few hundred pups produced on the island in 1962 to over 87,000 pups in 2016 (den Heyer et al. 2021), with a slowed growth rate observed beginning in the mid-2000s as the population likely approaches carrying capacity (Bowen et al. 2011). While smaller populations are expected to have lower trait heritabilities given the effects of drift and differential selection on traits, the consequences of bottlenecks on the heritability of traits varies (see Hoffmann et al. 2017). A recovering population may respond quickly to selection given an increase in additive genetic variance and heritability of particular traits; however, bottlenecks may also lead to inbreeding, resulting in lower trait values and loss of evolutionary potential (Taft and Roff 2012). The relationship between inbreeding and reduction in additive genetic variance of traits, as well as the possible effects of inbreeding depression on these fitness-related traits in the grey seal deserves further investigation.

5.5.2 Genetic architecture

Our genome-wide association analyses suggest a polygenic architecture underlying variation in the traits explored, as no significant peaks were detected in the GWAS and loci underlying trait variation had very small effect sizes. This finding agrees with multiple studies conducted on wild populations (e.g., Miller et al. 2018, Sim and Coltman 2019, Duntsch et al. 2020), such that it is becoming widely recognized that much variation in quantitative traits is influenced by many loci of small effect (Santure and Garant 2018). The grey seal maternal traits examined herein, such as lactation duration, boldness, and offspring weaning mass, are likely governed by complex and interconnected pathways involving morphological, behavioural, and physiological adaptations. As such, it is not unexpected that these traits might be controlled by many loci rather than by few loci of large effect. This knowledge of polygenic architectures underlying ecologically relevant traits provides important insight into the likely speed of adaptive responses and of the longer-term total response to selection (Duntsch et al. 2020). Combined with low heritability, such as that estimated for lactation duration, the rate of evolutionary response will likely be reduced.

Though we did not have a dense SNP panel, even studies containing large genomic datasets in systems such as wild ungulates and the great tit have not found SNPs that explain an appreciable proportion of heritable variation (e.g., Bérénos et al. 2015, Santure et al. 2015, and

Kim et al. 2018), highlighting that genome-wide association analyses have not met initial expectations (Gienapp 2020). Much variation in quantitative traits is left unaccounted for, potentially from contributions to phenotypic variance by parental effects, rare alleles, alleles of small effect, genetic interactions/correlations, and low linkage disequilibrium between the causal locus and marker genotyped (Manolio et al. 2009; Nelson et al. 2013). We recommend that complementary approaches be taken to further explore the genetic architecture of traits, such as chromosome partitioning and outlier analysis. Outlier analyses may prove especially useful in the effort to identify genomic regions underlying trait variation in systems such as the grey seal. In their study on the genomic demographic history of Northwest Atlantic grey seals, Cammen and colleagues (2018b) discovered outliers within the Sable Island population that could potentially be associated with traits under selection. Combining outlier analyses with our dataset may help in detecting putative outlier loci that, when mapped to an annotated representative genome, could identify potential candidate genes associated with advantageous phenotypic variation under selection, leading to local adaptation.

5.5.3 Future considerations

In addition to the population dynamic history of Northwest Atlantic grey seals, our sparsely dense marker dataset makes it difficult to discern between true polygenicity and whether adequate statistical power was obtained to detect true large-effect loci, if present. Although up to 460 individuals were used for analyses in our study, sample size may be a limiting factor, especially in the GWAS analysis. Simulations suggest that samples and markers numbering in the thousands and tens of thousands, respectively, may be necessary to accurately capture genomic relatedness and to elucidate the true genomic architecture underlying complex traits, even in species with small effective population sizes (Miller et al. 2018). Although SNP-based heritability estimates have improved precision over pedigree approaches, as observed even with a moderate number of SNPs (e.g., Malenfant et al. 2018 and Jamieson et al. 2020), these estimates may be underestimated when too few markers are used (Bérénos et al. 2014; Gervais et al. 2019). While we caution against overinterpretation of our results, the heritability estimates reported here did not vary in relation to sample size.

An interesting feature found amongst marine mammals is their typically low genomic diversity (Cammen et al. 2016), owing in part to bottlenecks resulting from an extensive history

of human exploitation for blubber and fur, but also attributable to various ecological characteristics such as their mating strategies, behaviour, and habitat use (e.g., Lancaster et al. 2007, Carroll et al. 2015). This begs the question: is there sufficient genetic variation in traits to detect genome-wide genotype-phenotype associations? Though the number of polymorphic markers we found is similar to that reported in a study on Weddell seals (*Leptonychotes weddellii*) (Miller et al. 2021), Cammen et al. (2018b) identified a higher number of polymorphic loci than the current study in Northwest Atlantic grey seals with which to perform genomic analyses. Using similar RADseq methodology, the authors found over 8700 polymorphic loci in three sampling locations and a total of 252 individual grey seals, including individuals from Sable Island used in the current study. This underscores how differences in sampling, laboratory protocols (e.g., restriction enzymes used), and bioinformatics (e.g., selection of Stacks parameters and application of filters and data pruning) can result in vastly different marker datasets (Andrews et al. 2016; Shafer et al. 2016; Paris et al. 2017). Nonetheless, final marker datasets used for analyses should be appropriate for the intended application. A dataset with rare alleles (MAF < 1%) and/or missing data, for instance, may be useful and informative for population studies such as demographic analyses (Linck and Battey 2019), but less informative to investigate the genetic basis of adaptive trait variation. We encourage emphasis placed on marker discovery and increasing SNP density in future studies. Increasing ease of whole genome resequencing combined with growing availability of pinniped genomic resources may provide the number of markers and information necessary to provide a finer-scale resolution of the genomic basis of adaptive traits in future studies.

Table 5.1: Animal models used to estimate additive genetic variance and heritability for each maternal trait measured in grey seals of Sable Island, Nova Scotia (Canada).

Response	Type of measure(s)	Fixed factors	Random factors
Maternal mass at parturition (kg)	Repeated	Age	ID, Year, Cohort
Primiparous dorsal body length (cm)	Single	Age, Cohort	NA
Age at first parturition (yrs)	Single	Year	NA
Pup birth mass (kg)	Repeated	Parity	ID
Pup weaning mass (kg)	Repeated	Age, Age^2, Sex, Wean day	ID, Year
Birth date (days)	Repeated	Age	ID, Year, Cohort
Lactation duration (days)	Repeated	Age, Wean day	ID, Year
Boldness	Repeated	Age	ID, Year

Table 5.2. Number of individuals and observations, range, mean, repeatability, heritability, and genomic inflation factor for eight traits measured in female grey seals of Sable Island, Nova Scotia (Canada).

Trait	N_{ind}	N_{obs}	Range	Mean (SD)	R^*	h^2 (SE)	λ (SE)
Maternal mass at parturition (kg)	200	320	88-261	175.2 (33.68)	0.45	0.13 (0.096)	0.94 (0.0013)
Primiparous dorsal body length (cm)	251	251	146-193	171 (8.03)	NA	0.38 (0.21)	0.96 (0.00081)
Age at first parturition (yrs)	449	449	4-10	5.8 (1.41)	NA	0.12 (0.12)	1.00 (0.0013)
Pup birth mass (kg)	203	322	9.5-32	20.7 (3.93)	0.04	0.16 (0.22)	1.02 (0.0014)
Pup weaning mass (kg)	458	4328	15.5-78.5	52.0 (8.32)	0.40	0.16 (0.049)	1.03 (0.0017)
Birth date† (date)	460	4727	18-68	46.5 (6.23)	0.48	0.16 (0.045)	0.98 (0.0008)
Lactation duration (days)	404	1672	5-28 days	17.1 (2.54)	0.23	0.08 (0.057)	0.96 (0.002)
Boldness	425	2991	1-6 (shy-bold)	3.1 (1.11)	0.58	0.19 (0.085)	0.98 (0.0016)

*Repeatability estimates adjusted for inclusion of random and fixed factors.

†Wean date used as proxy for birth date.

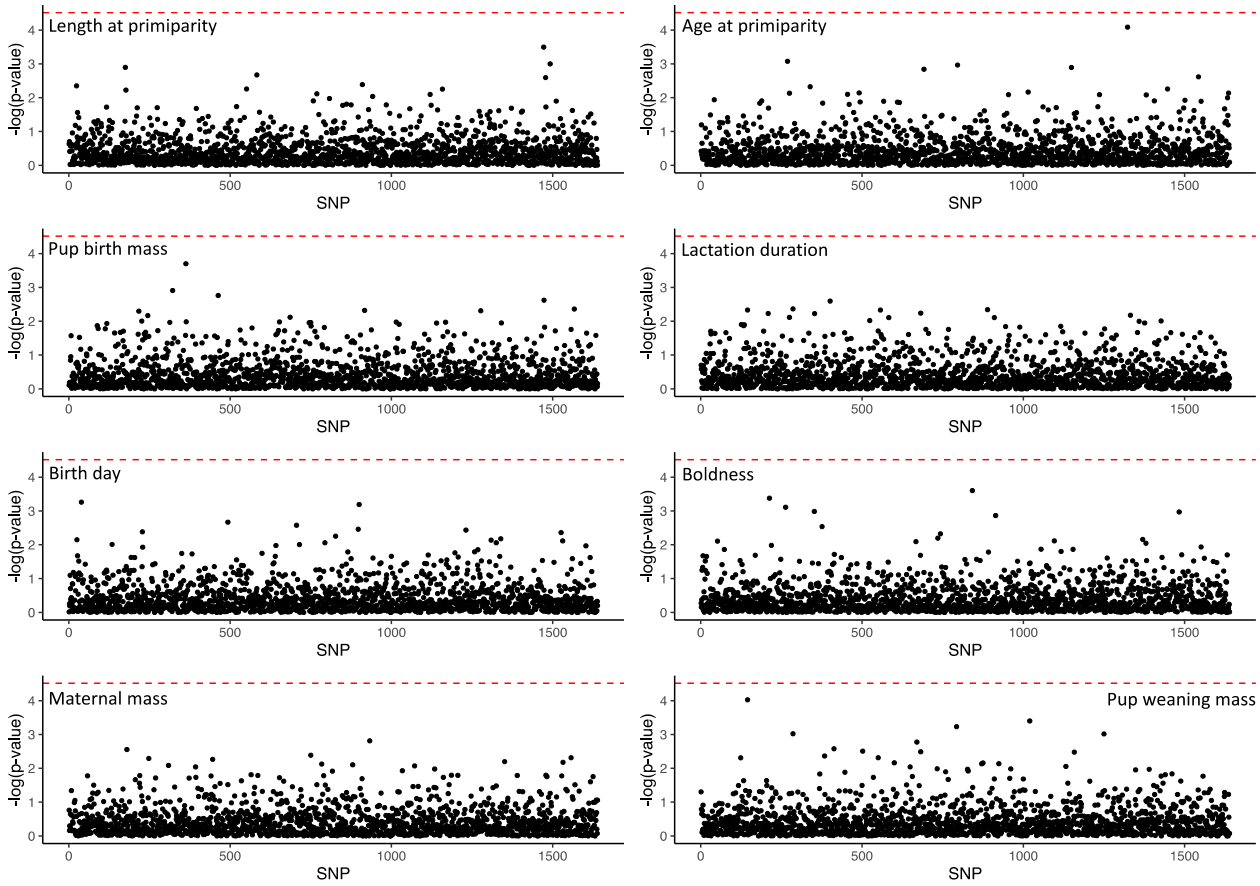

Figure 5.1: Manhattan plots for associations between SNPs and eight female grey seal traits: length at primiparity, age at primiparity, pup birth mass, lactation duration, birth day, boldness, maternal mass, and pup weaning mass. The red dashed line indicates a significance threshold using Bonferroni correction for the number of loci used (α = 0.05/number of loci).

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Appendices

D.1 Double digest restriction-site associated DNA sequencing library preparation protocol

DNA extracts were quantified using the Qubit Fluorometer (Life Technologies) and subsequently standardized to 20 ng/μl. We prepared double digest restriction-site associated DNA (ddRAD) sequencing libraries using the protocol as described by Peterson et al. (2012), but performed with minor modifications. Briefly, genomic DNA (12.5 μl of 20 ng/μl gDNA) was digested using 10 and 20 units of the restriction enzymes Sbf1 and EcoR1 (New England Biolabs), respectively, with 10X Cutsmart reaction buffer at 37°C for 2h, 65°C for 20 min, and a 4°C extension hold. Following the restriction digestion, we proceeded directly to ligating adaptors, wherein one of 16 individually 8-bp barcoded (i5 index) P1 adaptors and a single P2 adaptor were ligated to the SbfI and EcoRI sites, respectively. Ligation reactions occurred in 40ul volumes containing the restriction digest and 10X Cutsmart reaction buffer, 10mM ATP, 400U of concentrated T4 DNA ligase (New England Biolabs), and 5 ul of each working adaptor. Reactions were incubated at 22°C for 80 min, followed by 65°C for 20 min and a 4°C hold. Ligated samples were then pooled into groups of sixteen individuals, each having a unique barcode/i5 index, and subsequently cleaned using a QIAquick PCR Purification Kit (Qiagen, Valencia, California, USA) to remove excess adaptors and buffer. Library amplification occurred in 25ul reactions containing the purified library DNA, one of 12 P2 primers (2uM) (each P2 primer with a unique 8-bp i7 index), and Phusion® High-Fidelity PCR Master Mix (New England Biolabs). The PCR Master Mix consisted of: Phusion HF 5X buffer, 2mM dNTP, 10uM PCR1/P1 primer, and Phusion. Thermocycling parameters consisted of: denaturation at 98°C for 30 seconds, followed by 12 cycles of 98°C for 10 s, 54°C for 20 s, and 72°C for 1 min, extension at 72°C for 10 min and a 4°C hold. PCR product was pooled with respective P2 primers and purified again using the Qiagen PCR cleanup kit. A total of 35 ddRAD libraries were built and sequenced in three runs on a NextSeq 500 (Illumina, San Diego, CA, USA), with 75-bp single-end read sequencing. Libraries were prepared and sequenced at the University of Alberta's Molecular Biology Service Unit.

D.2 Additional tables

Table D.1: Breakdown of 485 grey seal females used for analyses according to year of birth (cohort).

Cohort	Number of Females
1970	3
1973	13
1974	12
1985	33
1986	42
1987	25
1989	14
1998	54
1999	66
2000	71
2001	67
2002	84

Table D.2: Descriptions of grey seal pup developmental stages used to assess female reproductive status and birth date for stage 1 pups on Sable Island, Nova Scotia (Canada).

Stage	Description	~ Days Old
1	Newborn with yellowish pelage still wet from birth fluids, loose skin folds	1-2 days
2	Pup with white lanugo, neck is defined with cylindrical body shape	2-5 days
3	Pup's lanugo is white to light grey, neck is no longer defined and body is fusiform shaped	5-13 days
4	Pup begins shedding lanugo	13-22 days
5	Pup has shed nearly all lanugo	> 22 days

Table adapted from Bowen et al. (2003)

Table D.3: Behavioural scores of grey seal females measured along the shy-bold continuum on Sable Island, Nova Scotia (Canada) from 2008-2019.

Score	Description
1	Shy; flees, quickly moves > 2 m away from researchers and pup
2	Shy; moves < 2 m away from researchers and pup
3	Intermediate; stays nearby and shows no boldness
4	Mild boldness; vocalizes and makes abrupt movements towards researchers
5	Moderate boldness; vocalizes, lunges towards researchers, displays open mouth threat
6	Extreme boldness; vocalizes, displays an open mouth threat, lunges and attempts to bite

Table D.4: Comparisons of top models to assess sources of variation on each female grey seal trait and associated AIC (Akaike Information Criterion) values. Support for the top model was provided by the lowest AIC, smallest ΔAIC (difference in AIC from top model), and highest AIC weight (*w*), while taking into account missing phenotypic data for putative fixed factors.

Trait	Covariates	AIC	ΔAIC	*w*
Primiparous length	Age	1660	0	0.328
	Year + Cohort + Age	1661.3	1.34	0.168
	Year + Cohort	1661.3	1.34	0.168
	Year + Age	1661.3	1.34	0.168
	Cohort + Age	1661.3	1.34	0.168
Primiparous age				
	Year	1541.9	0	1
	Cohort	1579.2	37.33	0
	Null	1588.7	46.85	0
Maternal mass				
	Age	2412.7	0	0.656
	Age + WeanDay	2414.8	2.1	0.229
	Parity	2416.8	4.1	0.084
	Parity + WeanDay	2418.9	6.18	0.03
	Null	2470.3	57.59	0
Wean date				
	Age	25693.6	0	0.994
	Null	25704	10.38	0.006
Pup birth mass				
	Parity + MatMass + Sex + WeanDay	1373.2	0	0.535
	Parity + MatMass + WeanDay	1374.1	0.81	0.357
	Parity + Sex + WeanDay	1377.7	4.42	0.059
	Parity + WeanDay	1378	4.77	0.049
	Age + MatMass + Sex + WeanDay	1400	26.77	0
Pup weaning mass				
	Age + Sex + WeanDay	28484.5	0	1
	Age + Sex	28517.8	33.3	0
	Age + WeanDay	28628.2	143.68	0
	Age	28676.9	192.47	0
	Sex + WeanDay	28911.1	426.68	0
Lactation duration				
	Age + WeanDay	7629.4	0	0.996

	Age + WeanDay	7640.3	10.84	0.004
	WeanDay	7673.9	44.48	0
	Null	7705.5	76.11	0
Boldness				
	Age + Habitat + WeanDay	6737	0	0.993
	Habitat + WeanDay	6746.9	9.91	0.007
	Age + WeanDay	6759.9	22.95	0
	WeanDay	6769.8	32.8	0
	Age + Habitat	7515.3	778.31	0

Table D.5: Repeatability estimates for morphological, life-history, and boldness traits in Sable Island female grey seals and associated model structures used for analyses.

Trait	N_{Ind}	N_{Obs}	R^*	95% CI	R_{Adj}	95% CI R_{Adj}	Random Factor	Fixed Factor
Maternal mass	62	160	0.56	0.37-0.69	0.45; 0.59	0.27-0.61; 0.45-0.74	ID	Age; Age, Age^2
Pup birth mass	73	190	0.15	0.01-0.27	0.04	0.0073-0.23	ID	Age, Age^2, MatMass
Pup weaning mass	434	4299	0.31	0.28-0.36	0.4	0.36-0.44	ID	Age, Age^2, Sex
Birth date	440	4707	0.71	0.68-0.74	0.48	0.38-0.57	ID, Year	Age
Lactation duration	322	1589	0.20	0.15-0.26	0.23	0.17-0.29	ID	Age, Wean Day
Boldness	399	2906	0.57	0.53-0.61	0.58	0.53-0.62	ID	Age, Habitat

*R, or agreement repeatability, estimated only accounting for seal ID as a random factor. Repeatability adjusted (R_{Adj}) for inclusion of fixed and random factors.

D.3 Additional figures

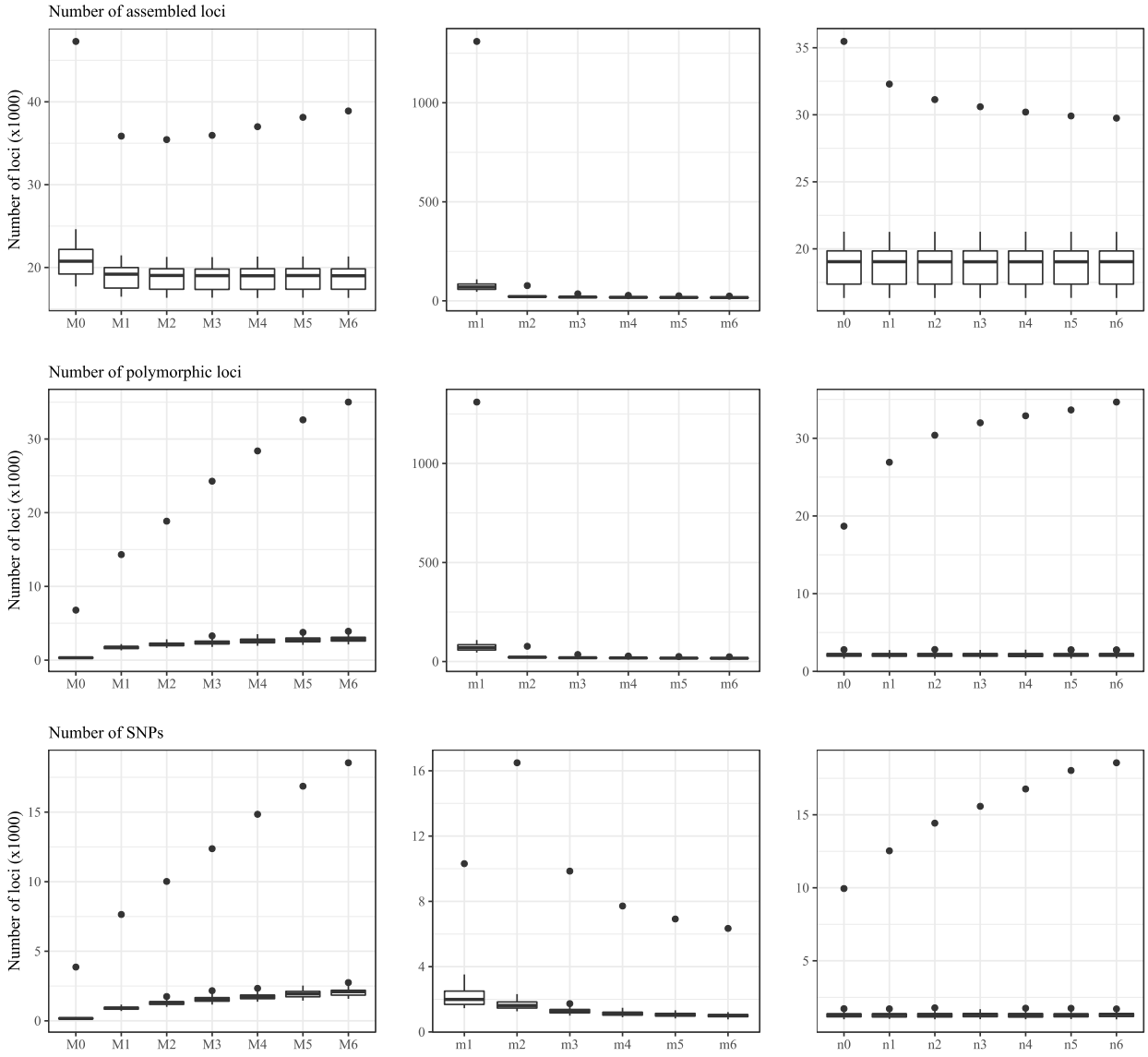

Figure D.1: Comparisons of effects from varying Stacks parameters *M*, *m*, and *n* on the number of assembled loci, polymorphic loci, and SNPs for 30 grey seal females of Sable Island, Nova Scotia (Canada).

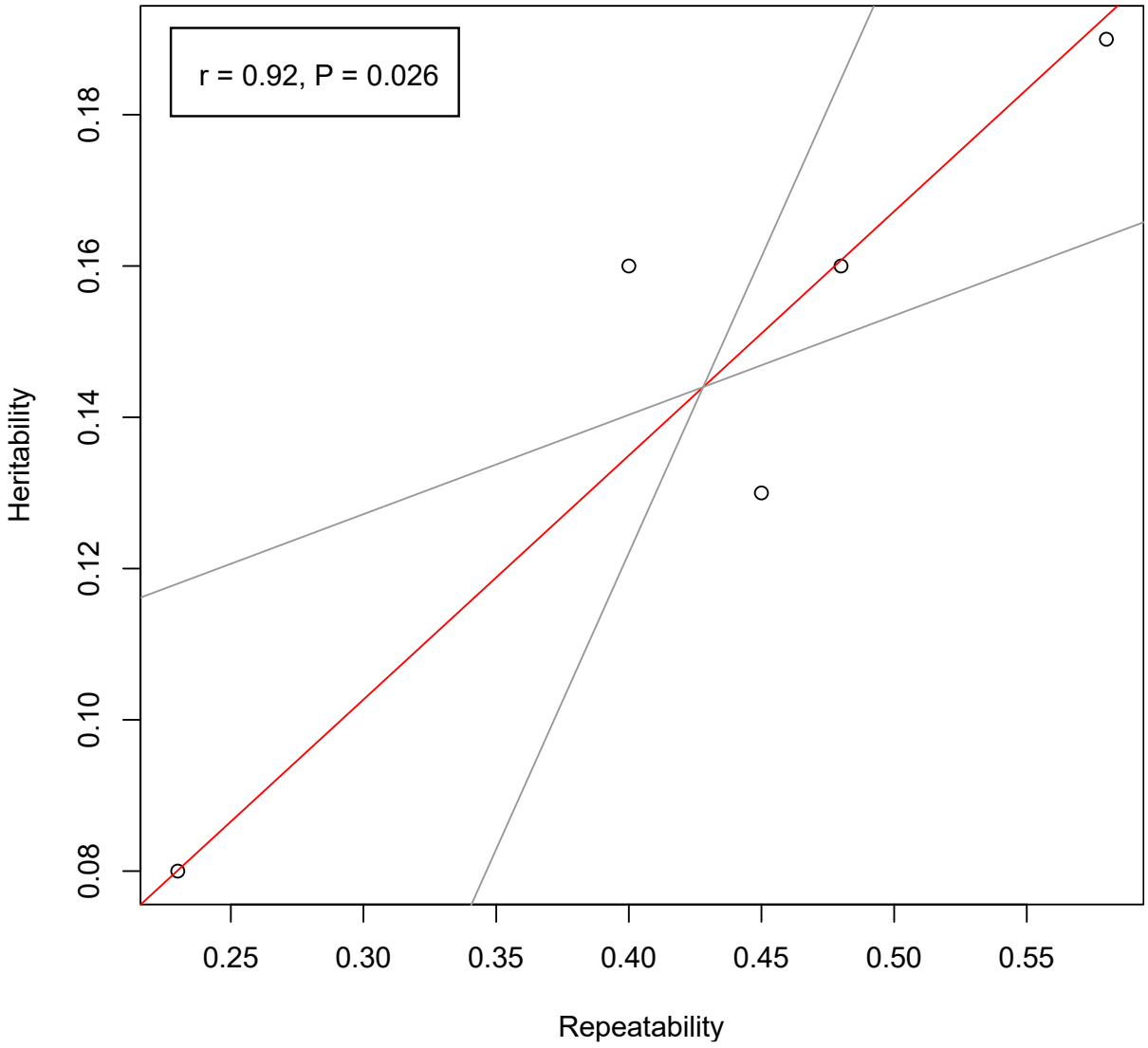

Figure D.2: Reduced major axis regression results depicting the relationship between significant repeatability and heritability estimates for repeat measured traits (lactation duration, pup weaning mass, maternal mass, birth date, and boldness) of female grey seals from Sable Island, Nova Scotia (Canada).

Chapter 6: General conclusion

6.1 Conclusion

My doctoral thesis was focused on examining the genetic basis of boldness and life-history traits in a free-ranging population of grey seals. This thesis integrated individual-based phenotypic records from a long-term field research program with DNA sequencing techniques, including a reduced-representation sequencing approach. My research applied quantitative genetic analyses to estimate the repeatable and heritable nature of maternal performance traits in Sable Island grey seals, and in doing so, provided evidence of a genetic component underlying the among-individual variation observed, as well as of the adaptive potential in the traits examined. In addition, I provided insight into the genetic architecture of these traits by using statistical techniques to test for associations between genetic markers and phenotypic values.

In **Chapter 2**, I conducted a literature review and meta-analysis to assess trends in analytical approaches used to investigate the relationship between genes and behaviour in natural systems, specifically candidate gene approaches, quantitative trait locus (QTL) mapping, and genome-wide association studies (GWAS). I determined the efficacy and perceived success of each approach and identified which behaviours and species have been commonly examined by researchers thus far. I found that most studies using QTL mapping and GWAS approaches reported a significant or suggestive effect between the trait of interest and genetic marker(s) tested, while over half of candidate gene accounts did not find a significant association. The top three behavioural categories examined included animal personality traits followed by reproductive and migratory behaviours. My findings showed that despite widespread accessibility of molecular approaches given current sequencing technologies, efforts to elucidate the genetic basis of behaviour in free-ranging systems has been limited.

In **Chapter 3**, I examined the effect of environmental and biological sources of variation on behavioural responses measured along the shy-bold continuum and further determined the repeatability of boldness in the Sable Island grey seal population. Over a nine-year period (2008-2016), 469 females were given a boldness score in response to a human approach and handling of her pup. There were age differences in boldness, such that younger females were generally less bold than older, more experienced females. I showed that boldness was highly repeatable between and within years. I further assessed sources of variation on offspring weaning mass and

found that young, bold females produced heavier pups than their shyer counterparts. Pups produced by bolder females of all age groups were on average ~2 kg heavier than pups of shy females. These results set the stage for further investigating the genetic basis of boldness in subsequent chapters.

In **Chapter 4**, I used a candidate gene approach to explore the association of genetic variants with repeated measures of boldness and reproductive performance (weaning mass and lactation duration) collected over an 11- and 28-year period, respectively, in Sable Island female grey seals. I isolated and re-sequenced five genes [dopamine receptor D4 (*DRD4*), serotonin transporter (*SERT*), oxytocin receptor (*OXTR*), and melanocortin receptors 1 (*MC1R)* and 5 (*MC5R*)] that have previously been linked with behaviour and fitness-related traits in primates, rodents, and avian species. I discovered single nucleotide polymorphisms (SNPs) that permitted testing for genotype-phenotype relationships at seven loci in three genes (*DRD4*, *SERT*, and *MC1R*). Using repeated measures data from 180 females having extreme shy-bold phenotypes, I tested for association between the seven loci and three maternal traits using mixed-effects models. I discovered that one locus within *SERT* was significantly associated with boldness (effect size = 0.189) and a second locus within *DRD4* with weaning mass (effect size = 0.232). Altogether, genotypes explained 6.52-13.66% of total trait variation. This study substantiated *SERT* and *DRD4* as important determinants of behaviour, and provided unique insight into the molecular mechanisms underlying maternal performance variation in female grey seals of Sable Island.

In **Chapter 5**, I used phenotypic records and genetic samples from the multi-decadal (1983-2020) Sable Island study on grey seals to perform quantitative genetic and genome-wide association analyses on eight female traits, representing morphological, life-history, and behavioural phenotypes. Using restriction site associated DNA sequencing, I obtained genotypic data for 476 females and determined that maternal traits examined had low to moderate heritability values (h^2 = 0.08-0.38). Genome-wide association analyses did not reveal any loci that were significantly associated with the traits examined, suggesting underlying polygenic architectures. This study suggests an evolutionary capacity for traits to respond to selection in Sable Island grey seals.

When I started my PhD studies, generating genome-wide data was increasingly feasible for any species, permitting addressing long-standing evolutionary biology questions. In addition,

there was a growing acknowledgement within the scientific community of the existence and eco-evolutionary consequences of animal personality; moving away from a reductionism dogma of behaviour analysis to an interest in unravelling the mechanisms driving and conserving behavioural variation (Bell 2017). In this thesis, I made contributions to the animal behaviour literature by building upon previous work that evaluated the repeatable (Bell et al. 2009) and heritable (Dochtermann et al. 2019) nature of animal behavioural diversity. The literature review/meta-analysis presented in Chapter 2 filled a gap in the literature by summarizing efforts made to explore the genetic/genomic architecture of repeatable and heritable behavioural traits observed in natural populations as reviewed by Bell et al. (2009) and Dochtermann et al. (2019), respectively. This work notably revealed that while there has been an uptick in the number of behavioural studies attempting to link phenotype with genotype, research has been limited in taxonomic breadth and scope.

In Chapter 3, I was among the first to demonstrate evidence of an animal personality signal in a wild pinniped population, and showed that boldness variation was linked with a component of reproductive success in the population. With inference of a genetic basis to boldness in grey seals (Chapters 3 and 4), I moved the behavioural candidate gene literature on free-living mammals beyond non-human primates and rodents (Bubac et al. 2020), and provided supported that certain genes of high interest (e.g., *SERT* and *DRD4*) explain a proportion of variation in natural behaviour and reproductive performance. Chapter 4 also provided a benchmark for estimates of standing genetic variation in potentially adaptive genes in the Sable Island grey seal population. The heritabilities estimated in Chapter 5 are a first for a phocid, and among few other studies that have assessed the quantitative genetics and genomic architecture of fitness-related traits in a wild marine mammal population (e.g., Authier et al. 2011, Malenfant et al. 2018). Collectively, Chapters 3-5 shed light on the genetic basis of behavioural and life-history traits specific to grey seals of Sable Island – likely a large source to other growing colonies recolonizing their historical range in Canada and the United States (Wood et al. 2011; den Heyer et al. 2021).

The work presented in this thesis has not been met without its challenges. Difficulties experienced in obtaining adequate coverage and recovery of polymorphic loci are not unique to the collective studies presented herein, but represent shared obstacles experienced when attempting to elucidate the genetic basis of complex traits in the wild (e.g., Edwards et al. 2015;

Holtmann et al. 2016; Madlon-Kay et al. 2018; Miller et al. 2018). When I began sequencing work in 2017, and to my knowledge, only genomic resources made publicly available for phocids (i.e. true seals) existed for the Weddell seal (*Leptonychotes weddellii*) - a species that grey seals shared a common ancestor with approximately 15 million years ago (Fulton and Strobeck 2010). In addition, RADseq resources specific to the Northwest Atlantic population of grey seals had not yet been published (Cammen et al. 2018b). The astounding pace of genomic technological advancement, combined with widespread accessibility and reduced costs associated with sequencing, has now resulted in a draft genome for not only the grey seal, but also for the spotted seal (*Phoca largha*), harbor seal (*Phoca vitulina*), bearded seal (*Erignathus barbatus*), Northern elephant seal (*Mirounga angustirostris*), and Hawaiian monk seal (*Neomonachus schauinslandi*) (dnazoo.org; Dudchenko et al. 2017). These genomes promise to facilitate marker discovery in free-living phocid populations, and once annotated, will enable and aid in the identification and finer-scale resolution of relationships between candidate loci and phenotypic variation.

This dissertation provided insight into the genetic basis of quantitative traits in a natural population, while highlighting future research potential. First, I recommend extension of quantitative genetic analyses to include estimation of genetic correlations between the maternal performance traits examined. Bivariate models can be used to estimate these covariances and assess potential trait responses to and rate of indirect selection (Roff 1996), which may influence the reproductive heterogeneity observed among grey seal females (e.g., Badger 2020). Second, genomic inbreeding coefficients could be estimated to assess whether inbreeding depression has an effect on the reproductive performance traits in female grey seals of the Sable Island population. SNP-based inbreeding coefficients have proven valuable in examining the fitness costs of inbreeding in free-living populations (e.g., Huisman et al. 2016), but insofar has been limited to few systems.

Associating phenotype with genotype has low replicability success across studies (Schielzeth et al. 2018); therefore, I recommend validation studies be performed within the Sable Island population as well as across breeding colonies of grey seals. The North Rona, Scotland breeding colony of grey seals, for example, may be valuable for comparison as this colony has also been the focus of studies aligning with the identification of consistent individual differences in various behavioural and maternal performance traits, spanning multiple years (Twiss et al.

2010; Twiss et al. 2012; Robinson et al. 2019). While candidate gene studies are limited in scope, this approach remains a logical and accessible one that has been successful in providing understanding of the conservation and general role of gene-trait associations in various species, as well as of local adaptive phenotypic divergence (van Oers and Mueller 2010). As such, resequencing promising genes, such as *OXTR,* that were not fully sequenced in Chapter 4, and expanding sequencing effort to include additional major candidate genes including monoamine oxidase A (*MAO-A*; associated with aggression, stress, and boldness) and arginine vasopressin receptor 1A (*AVPR1A*; associated with aggression, boldness, and reproductive behaviour) within and across populations may provide further insight into the genetic basis of trait variation. Lastly, I recommend using complementary genome-wide methodologies to further validate, or otherwise, the polygenic architecture underlying trait variation. This can be achieved by methods such as chromosome partitioning, wherein polygenicity is supported if more than one chromosome and/or a large chromosome having more genes is found to explain a large proportion of variance (Yang et al. 2011). Outlier analyses may also aid in the detection of loci that, when mapped to an annotated representative genome, could identify potential genetic regions associated with advantageous phenotypic variation under selection, leading to local adaptation (e.g., Tigano et al. 2017, Wellband et al. 2019).

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