Genetics of behavioral evolution following island colonization

By

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Abstract

Behavior is a fundamental aspect of fitness in animals and plays a unique role in shaping the interaction between organisms and their environment. Despite the importance of behavior in adaptation, the genetic basis of behavioral evolution in natural populations is poorly understood. On islands, animals are often bolder and more exploratory, changes predicted by known ecological shifts in insular environments. In this thesis, I characterize the genetic basis of island-associated behaviors in a unique house mouse population from Gough Island (GI). I begin by describing historical approaches to the study of behavior and how giant mice from GI serve as a model system to genetically characterize rapidly evolved behaviors. I show that GI mice are bolder and more exploratory than mainland conspecifics but only when a predator cue is absent, suggesting behavioral evolution is context specific. Then, using crosses between strains of GI mice and mainland mice, I report quantitative trait loci (QTL) contributing to the evolution of insular behaviors. I find that evolved boldness and exploratory tendencies in GI mice are genetically separable. In each behavioral category, I reveal that QTL effects can be temporally dynamic during behavioral assays and can be influenced by age, sex, and environmental context. I then extend the characterization of QTL to include those arising from social partners. I describe four QTL that are associated with behavior and body size of cagemates, a rare description of loci conferring indirect genetic effects in a natural population. These indirect QTL are distinct from direct QTL but have similar effect sizes. My findings demonstrate that indirect QTL can be mapped using traditional approaches that detect direct QTL, highlighting an untapped potential in standard mapping populations. I conclude by summarizing findings and emphasizing the need to investigate roles of life history and environmental context on behavior. I advocate for fine mapping of the QTL I discovered and aggression profiling in GI mice to further characterize behavioral evolution in this system.

Introduction

Adaptation of populations to local environments is a fundamental source of phenotypic diversity in nature. Environmental changes can drive the evolution of unique traits, such as cryptic coloration in peppered moths or pollution resistance in Atlantic killifish in response to urbanization (Cook and Saccheri 2013; Reid *et al.* 2016; Diamond *et al.* 2022). Adaptations arise through the alternative fitnesses of phenotypic variations, a mechanism first proposed by Darwin and Wallace (1858). For selection to occur, the underlying traits must be heritable. Mendel described how traits can be inherited through particulate factors (Mendel 1866), even when the resulting phenotypes are on continuous scales (Fisher 1930). Natural selection can use these discrete genetic factors to bring populations to adaptive optima (Fisher 1930; Orr 2005). With the advent of genomic sequencing, genetic variants underlying adaptive traits can be identified by testing associations between genotypes and phenotypes (Phifer-Rixey *et al.* 2015; Gienapp *et al.* 2017; Reid *et al.* 2021; Bomblies and Peichel 2022). This reductionist approach is a powerful way to determine the history of adaptive evolution. However, it simplifies organisms down to individual traits under examination. A holistic understanding of adaptation requires a broader perspective of organismal complexity.

The selective advantage of each trait is determined by its connection to fitness. This remarkable fact allows theoreticians to evaluate all sources of genetic variation on the same axis. However, organisms are multi-dimensional entities whose realized fitness is determined by the cumulative and/or interactive variation across all traits (Lande and Arnold 1983; Phillips and Arnold 1989; Schluter and Nychka 1994). Most investigations focus on only one trait, typically whatever is most eye-catching to the investigator. This narrow perspective can miss important aspects of the adaptive process. Genetic correlations between different adaptive traits can influence evolutionary trajectories (Schluter 1996; Conner *et al.* 2011). Additionally, certain parameters that govern adaptive evolution, such as constraint and pleiotropy, can only be evaluated in a multivariate framework (Brodie 2017). For example, particular combinations of coloration and escape patterns provide higher fitness in garter snakes under

predation, but each trait is individually uncorrelated with survivorship (Brodie 1992). In song sparrows, selection prefers a specific wing length to body mass ratio (Schluter and Nychka 1994). Investigating naturally adapted populations across multiple trait categories can provide a richer view of the adaptive process than is typically presented.

Behavior is a fundamental trait that can determine broadscale evolutionary trajectories of animals (Wcislo 1989). Behavior is often described as the actions of an organism in response to stimuli and thus provides a unique connection between an animal and its environment (Ben-Shahar 2017). Baldwin (1902) proposed that the initial success of animals in new environments is facilitated by behavioral responses with phenotypic changes across other traits occurring subsequently. This idea posits that behavior acts as a gateway for adaptation (Mayr 1963; Wcislo 1989; Futuyma 1998). Additionally, behavior plays a key role in social evolution (Bourke 2011). The social environment can have widespread effects and shape phenotypic diversity in any population or experiment where conspecifics can interact, a pervasive influence that is often ignored. The connections between behavioral variation, fitness, and relatedness of interacting partners contribute to evolutionary novelties such as altruism (Rodrigues and Gardner 2022), division of labor (Page and Erber 2002; Canciani *et al.* 2019), parent-offspring conflict (Trivers 1974; Godfray 1995), and aggression (Maynard-Smith and Price 1973; Wilson *et al.* 2009). Because of its central role in evolution, behavior has been a key topic of interest since the beginning of evolutionary study. Darwin’s “The Expressions of the Emotions in Man and Animals” (1872) was dedicated solely to behavioral diversity across animals. Previous studies of behavioral evolution primarily consisted of two disciplines: 1) ethology, the examination of stimuli that elicit behavioral responses, and the behavioral profiles that animals adopt in nature to improve fitness, and 2) behavioral genetics, the dissection of molecular mechanisms underlying behavior in humans and model organisms. Studies that unite these two subfields, investigations that examine the genetic architectures underlying evolved behaviors in nature, are rare (Niepoth and Bendesky 2020).

Insights into the genetic basis of behavioral evolution begin with observations of natural diversity. Human assessment of animal behavior predates modern history (Maxson 2006). Domestication of animals resulted in sweeping changes across behavioral and neurobiological traits (Beckman *et al.* 2022) suggesting ancient humans recognized behavioral variation and selectively bred for desired personalities. Darwin brought behavior to the forefront of scientific study when he discussed the tameness of domesticated animals and peculiar behaviors of domestic pigeons in the first chapter of "The Origin of Species" (Darwin 1859). Darwin also proposed that behavioral evolution can cause remarkable modifications between the sexes, leading to the development of unusual colorations and weaponry used in displays of courtship and territorial aggression respectively (Darwin 1871). In the twentieth century, Karl von Frisch, Nikolaas Tinbergen, and Konrad Lorenz pioneered the field of ethology by describing how discernable stimuli can elicit predictable behavioral responses (Burkhardt 2005; Dhein 2021). Recent investigations extend Darwin's initial studies of behavior by asking what ecological shifts drive behavioral adaptations in nature.

Mathematical modeling based on evolutionary theory is used to show how naturally observed behaviors correspond to strategies that favor survival and reproductive success in different environmental conditions. These models often consider integrated networks of behaviors such as personalities or syndromes. Animal personality is described as the consistency of decisions an individual makes across time and contexts (Cabrera *et al.* 2021). The form and stability of personality explains the degree to which behavioral profiles are adapted to their native environment and the responsiveness of behaviors to environmental changes. Since different behaviors can have similar connections to fitness depending on a given life history strategy, we expect different aspects of personality to correlate with each other causing behavioral syndromes (Sih *et al.* 2004). For example, animals investing in rapid growth and earlier reproduction are more likely to take risks leading to increased boldness and increased aggression (Stamps 2007; Biro and Stamps 2008). The causes of mortality, which often involve

predation, are central in determining behavioral strategies (Sih *et al.* 2004; Stamps 2007). As such, a common question is the expected rate of foraging under environments with different rates of predation (Pyke 1984; Stephens and Krebs 1986). These foraging-risk ratios (Brown and Kotler 2004) predict how animals should behave to maximize resource acquisition for greater reproduction while minimizing mortality. Mathematical modeling can help explain why behavior and ecological variables covary but ignores genetic variation which is necessary for evolution.

Attempts to understand the genetic basis of behavior began with Darwin's contemporary and cousin, Francis Galton (Maxson 2006). Galton measured patterns of similarity between family members and concluded that behaviors can be heritable (Galton 1869). Following the emergence of molecular genetics, the ability to draw direct connections between genetic variants and behavior was possible. Seymour Benzer pioneered this field through experiments which determined that behaviors in fruit flies, like other mutable phenotypes, can be mapped and characterized using traditional forward genetic screens (Konopka and Benzer 1971; Weiner 1999). These experiments proved that variants in individual genes can cause large shifts in behavior. Genetic variants associated with behavior are increasingly being mapped across model organisms (Kendler and Greenspan 2006). Moreover, the molecular machinery underlying conserved behaviors seems to be similar across taxa (Reaume and Sokolowski 2011). Of particular interest are laboratory house mice where many quantitative trait loci (QTL) have been mapped that are associated with personality traits analogous to humans (Flint 2003). Evidence so far suggests that behaviors have largely similar genetic architectures to other kinds of quantitative traits, with most loci having small to moderate effects (Flint and Mackay 2009; Peichel and Marques 2017). However, different behavioral categories can show unique architectures (York 2018). At the same time, the effect of a locus on multiple behaviors can cause overlap in the sets of genes contributing to distinct aspects of personality (van Oers and Mueller 2010; Hoekstra and Robinson 2022). Investigations of the genetic causes of behavioral variation in model organisms have been essential to furthering our

understanding of human behavioral disorders (Sokolowska and Hovatta 2013; Hohoff 2009). Similar studies are needed in natural populations to understand the process of behavioral evolution in the wild.

Genetic studies of behavior using natural populations are relatively rare (van Oers and Mueller, 2010). The limited number of studies so far appear to recapitulate aspects of the genetic architecture observed in laboratory populations. For example, Weber *et al.* (2013) dissected the genetic architecture of differences in burrow shapes and sizes between oldfield mice (*Peromyscus polionotus*) and deer mice (*Peromyscus maniculatus*) and found that burrow size is complex with many contributing loci while the presence of an escape tunnel is controlled by a single, dominant locus. Other findings come from research in sticklebacks which have adapted to freshwater habitats showing remarkable transitions from their marine ancestors (Reid *et al.* 2021). Common garden experiments revealed that multiple, independent freshwater populations of sticklebacks have heritable increases in boldness and risk-taking behaviors likely due to the lack of predators in their native environment (Fraimout *et al.* 2022). Individuals from benthic lake populations are typically solitary and show reduced schooling behavior (Wark *et al.* 2011), an evolutionarily ancient defense against predators. Genetic crosses between benthic and marine sticklebacks revealed that schooling ability, tendency, and motivation are genetically separable traits (Greenwood *et al.* 2013; Greenwood *et al.* 2015). Despite this complexity, a single gene (*Eda*) was identified in causing much of the difference in schooling ability between the two populations (Greenwood *et al.* 2016). This locus also controls transitions in morphology in sticklebacks (Mills *et al.* 2014) suggesting the role of pleiotropy in adaptation can extend across trait categories. The previously described examples are important steps in understanding behavioral evolution, but the number and taxonomic breadth of studies using natural populations is still limited. Furthermore, sample sizes for genetic mapping experiments like those in sticklebacks are typically small, reducing the power to detect QTL and overestimating effects in detected loci (Beavis, 1998). Additionally, genetic variation arising from social partners (i.e., indirect genetic effects) is largely unexplored but can have significant impacts

on evolutionary trajectories (Moore *et al.* 1997; Wolf *et al.* 1998; Fisher and McAdam 2019). Extensive characterization of common patterns in behavioral evolution through large genetic crosses has the potential to fill important gaps in our knowledge of the adaptive process.

Populations on islands are paradigms of evolutionary patterns. The extreme phenotypes of insular populations inspired many of Darwin's theories about natural selection (Losos and Ricklefs 2009). Island and mainland environments are often drastically different, forcing island colonizers to adapt simultaneously to many ecological changes (Grant 1998; Losos and Ricklefs 2009). Biodiversity on islands is typically lower with a notable absence of predators and resources common on the mainland (Losos and Ricklefs 2009; van der Geer *et al.* 2011). Islands can also be colder at higher latitudes (Chown and Smith 1993) or experience harsher weather (Whittaker and Fernández-Palacios 2007). These changes drive phenotypic evolution across many trait categories including body size (Foster 1964; Van Valen 1973) and behavior (Blumstein and Daniel 2005; Cooper *et al.* 2014; Gavriildi *et al.* 2022) in patterns common enough to be described by singular terms. The "Island Rule" declares that small organisms become larger following island colonization while large organisms become smaller. "Island tameness", first described by Darwin (1840), refers to the decrease in antipredator behavior accompanying island evolution. The correlated changes in insular populations across all traits have been synthesized into a common phenomenon deemed the "island syndrome" (Adler and Levins, 1994). The combination of clear ecological drivers and prevalence of corresponding adaptations makes insular populations a powerful system to test predictions of evolutionary processes. However, while the ecology of islands and phenotypic diversity of insular animals are well-described, a genetic understanding of insular adaptations is limited.

A prominent example of rapid evolution following island colonization is Gough Island house mice. Gough Island (GI) is a remote volcanic island in the south Atlantic Ocean. The nearest mainland, South Africa, is over 1750 kilometers away, leading to a unique species composition. There is no human

presence on the island outside of a small weather station (Heaney and Holdgate 1957). The island's most important ecological function is its use as a nesting ground for seabirds, especially the Atlantic petrel and Tristan albatross (Ryan *et al.* 2001). House mice (*Mus musculus domesticus*) were first noted on GI in the late 19th century (Verril 1895) though estimates of demography from genomic data suggest colonization could have occurred earlier (Payseur and Jing 2021). GI mice are the only mammalian species on the island (Jones *et al.* 2003) and show remarkable patterns of phenotypic evolution corresponding to predictions of island ecology. GI mice are the largest wild house mice in the world (Gray *et al.* 2015). They also live in very densely populated conditions, around 224 mice per hectare (Rowe-Rowe and Crafford 1992). Perhaps most unusual is the novel predatory behavior these mice show towards seabirds. GI mice predate on seabird chicks, killing roughly 2 million chicks/eggs per year (Caravaggi *et al.* 2019). The prevalence of this predation drove efforts to eradicate the mice which have reduced, but not eliminated, the population (Samaniego-Herrera *et al.* 2022). Though diminished in the wild, inbred strains of GI mice are available for use in genetic dissection of island evolved traits.

The morphological differences between GI mice and mainland strains have been extensively characterized. Genomic tools and phenotypic assays developed for laboratory house mice facilitate extensive profiling of identical traits in GI mice. QTL for body size, skeletal size, and skeletal shape between GI mice and mainland mice have been identified (Gray *et al.* 2015; Parmenter *et al.* 2016). These traits were shown to be genetically complex though certain loci are major drivers of total mass and global skeletal expansion. Transcriptomic analysis also revealed extensive modification of gene regulation in important metabolic organs within GI mice that is likely connected to their extreme size (Nolte *et al.* 2020). The masticatory apparatus of GI mice is stronger than in mainland mice (Parmenter *et al.* 2020) and subsequent studies mapped QTL for mandibular shape (Parmenter *et al.* 2022), a rare combination of studies examining both structure and function of an evolved trait. The genetic characterization of morphological evolution in GI mice is among the first for insularly evolved traits.

These mice have the potential to reveal another first for evolutionary study, an initial genetic portrait of "island tameness". Examination of both behavior and morphology in the same population would provide a rare comprehensive picture of how multiple traits coevolved to the same ecological shifts.

This dissertation describes evolved behaviors of GI mice and provides a genetic characterization for this instance of "island tameness". In Chapter 1, I utilize common assays for anxiety in laboratory house mice to show that GI mice are bolder and more exploratory than a mainland conspecific. I also show how these behaviors are sensitive to environmental context and life history parameters. In Chapter 2, I map QTL for these behaviors and find that different behavioral categories are genetically distinct and have complex genetic architectures. I reveal that many QTL, like the behaviors they associate with, are context specific. In Chapter 3, I explore the importance of the social environment on the evolution of both body size and behavior in GI mice. I map QTL that are associated with body size and behavior of cagemates, a rare characterization of indirect genetic effects. To conclude, I summarize findings and discuss opportunities for further insights using this unique population.

Chapter 1

Evolution of boldness and exploratory behavior in giant mice from Gough Island

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Contributions were as follows: JAS helped design the study, carried out the behavioral testing, performed the video analysis, conducted the statistical testing, and drafted the manuscript; MJN conceived of the study, helped design the study, and critically revised the manuscript; BAP helped design the study and critically revised the manuscript.

Abstract

Island populations are hallmarks of extreme phenotypic evolution. Radical changes in resource availability and predation risk accompanying island colonization drive changes in behavior, which Darwin likened to tameness in domesticated animals. Although many examples of animal boldness are found on islands, the heritability of observed behaviors, a requirement for evolution, remains largely unknown. To fill this gap, we profiled anxiety and exploration in island and mainland inbred strains of house mice raised in a common laboratory environment. The island strain was descended from mice on Gough Island, the largest wild house mice on record. Experiments utilizing open environments across two ages showed that Gough Island mice are bolder and more exploratory, even when a shelter is provided. Concurrently, Gough Island mice retain an avoidance response to predator urine. F1 offspring from crosses between these two strains behave more similarly to the mainland strain for most traits, suggesting recessive mutations contributed to behavioral evolution on the island. Our results provide a rare example of novel, inherited behaviors in an island population and demonstrate that behavioral evolution can be specific to different forms of perceived danger. Our discoveries pave the way for a genetic understanding of how island populations evolve unusual behaviors.

Introduction

Organisms that colonize islands experience unique environmental challenges that require novel solutions (Losos and Ricklefs 2009). New kinds and distributions of resources on islands can select for new foraging strategies (Williamson 1981; Grant 1998, 1999). For many island colonizers, a loss of predators alleviates the need for behavioral or morphological defenses (Williamson 1981; Blumstein and Daniel 2005; Dmitriew 2011). If resources are difficult to acquire and predatory risk is absent, the optimal foraging-risk ratio is highly skewed, favoring exploration and boldness (Brown and Kotler 2004; Creel and Christianson 2008; Elliott *et al.* 2017). Island populations, therefore, provide opportunities to test hypotheses about the evolution of behavior in novel environments.

Populations that spread to islands often display behavioral changes. Many populations lose anti-predator behaviors (Blumstein and Daniel 2005). Some island inhabitants are more easily approached by humans, a phenomenon Darwin called “island tameness” (Darwin 1840; Cooper *et al.* 2014). In some island rodents, territoriality and conspecific aggression are reduced compared to mainland populations (Halpin 1981; Baier and Hoekstra 2019). Behavioral responses to island environments sometimes occur in the context of other phenotypic shifts. New behaviors are often accompanied by transitions in life history and morphology (Foster 1964; Halpin 1981; Berry 1992) in a pattern referred to as the “island syndrome” (Adler and Levins 1994).

Although behavior is suspected to be an important component of adaptation to island conditions, the question of whether island populations harbor heritable changes in behavior has rarely been answered (Jolly et al. 2018; Baier and Hoekstra 2019). Demonstrating that behavior has evolved on islands requires evidence of genetic change in behavioral traits.

A promising organismal system for testing hypotheses about behavioral evolution in response to island environments is found on Gough Island, a remote volcanic island in the middle of the South Atlantic Ocean. Despite the remote location of Gough Island, over 2,700 miles from the nearest mainland, house mice (Mus musculus domesticus) colonized the island, probably via sealing ships from western Europe a few hundred generations ago (Verrill 1895; Wace 1961; Gray *et al.* 2014). In a remarkable phenotypic transformation, these mice evolved a body size twice that of their mainland counterparts (Rowe-Rowe and Crafford 1992; Jones *et al.* 2003). Laboratory-born offspring of Gough Island mice (hereafter "GI mice") maintain their unusual size, confirming that this morphological distinction has a genetic basis (Gray *et al.* 2015; Parmenter *et al.* 2016). On Gough Island, mice live without the predators and without the human commensals they commonly exploit for food and shelter on the mainland (Rowe-Rowe and Crafford 1992; Phifer-Rixey and Nachman 2015). The diet of GI mice is highly variable and seasonal, with invertebrates (mainly earthworms) and seeds being the most stable food source (Jones *et al.* 2003). During the winter season, GI mice predate on endangered seabird populations that use the island as a nesting ground, leading to the deaths of an estimated 2 million chicks and/or eggs per year (Caravaggi *et al.* 2019). The combination of increased body size, novel consumption of birds, loss of predatory danger, and removal of human commensals predicts the evolution of increased exploration and boldness in GI mice. Because GI mice are western European house mice (*Mus musculus domesticus*), the same subspecies as the laboratory mouse (Gray *et al.* 2014), established methods in biomedical research can be applied to profile behavioral evolution.

In this article, we use GI mice to examine recent behavioral evolution on an island. By exposing juvenile and adult mice to novel environments with different levels of perceived risk, we uncovered multiple lines of evidence that GI mice are bolder (i.e., less anxious) and more exploratory than mice from a mainland reference strain. The detection of these differences among inbred strains raised in a common environment demonstrates that they are inherited. Our findings indicate that GI mice evolved

enhanced boldness and exploration (evidenced by the distance traveled during a behavioral test) over a short timescale and in concert with other substantial phenotypic changes. This work lays the foundation for identifying the genetic changes responsible for behavioral evolution in organisms that colonize islands.

Methods

Mouse Strains and Husbandry

Inbred strains of GI mice and mainland mice were used throughout this study. In 2009, GI mice were live-caught and shipped to the University of Wisconsin School of Veterinary Medicine Charmany Instructional Facility, where a breeding colony was established (Gray *et al.* 2015). GI mice for this study belonged to a strain maintained for 21–23 generations of brother-sister mating. At this stage of inbreeding, we expect most of the genome to be homozygous, allowing us to treat individual mice as replicates of the same genetic background. Mainland mice belonged to the WSB/EiJ inbred strain founded from breeding pairs caught in Maryland (purchased from the Jackson Laboratory, Bar Harbor, ME) and were maintained in the same colony as GI mice for the same time period. GI mice and house mice from the eastern coast of North America are likely descended from Western Europe (Gray *et al.* 2014; Phifer-Rixey *et al.* 2018), though the geographic locations of source populations are unknown. F1s were generated by crossing mice from the GI strain and the mainland strain in both maternal directions. All mice were housed in micro-isolator cages with corn cob substrate (1/8th inch; The Andersons Lab Bedding), with ad libitum access to food (Envigo 2020X Teklad Global Diet) and water. Breeders were provided with a higher fat chow (Envigo 2019 Teklad Global Diet) and a red mouse igloo (Bio Serv). All cages were provided with nesting material and irradiated sunflower seeds (Envigo). Cage changes occurred every 6–8 days or, in the case of a new litter, 10 days after parturition. The colony was kept in a temperature-controlled room (20–23°C) under a 12-hour light/dark cycle.

Mice used for behavioral testing were weaned 20–21 days after parturition and housed with one littermate of the same sex to reduce behavioral effects of within-cage hierarchies (Horii *et al.* 2017). All mice were weighed to the nearest tenth of a gram during cage changeouts and after the last behavioral assay (i.e., weekly from ages 4–10 weeks old +/− 1 day).

Behavioral Assays

All behavioral assays were conducted in a room separate from the main colony. Each subject was tested four times with the following regimen (see Fig. 1A): open field (4 and 8 weeks old +/− 1 day), light/dark (9 weeks old +/− 1 day), and predator cue (10 weeks old +/− 1 day). All tests were conducted during the light phase of the light/dark cycle under white, fluorescent light. Females were scored for stage of estrous cycle according to Caligioni (2009) on the same day as testing, beginning with the second open field test. Twelve females (all from the mainland strain) were in estrous the same day as testing. These animals fell within the range of values for each trait measured in that strain and were included in the final analysis. The order of subjects tested within litters each day was randomized. Each test began by bringing the subjects' cage into the room for a 30-minute acclimation period. The experimenter remained in the room out of sight of the subjects throughout the acclimation and testing periods except for transfer to and from the arena. The relevant arena for each test (see Fig. 1B–D for examples) was placed in the center of the room next to a movable cart where a computer and video recording hardware were located. The arena was cleaned with 70% ethanol after each test and at least five minutes was allowed for the ethanol to evaporate before testing a new subject. For the light/dark test the floor panels were removed and shaken to evaporate the ethanol instead of using a five-minute waiting period. All tests were video recorded using Debut Video Capture Software v 5.33 at default settings with Focus set to 0. A Logitech HD Pro Webcam C920 was positioned directly above the center of the arena. Two videos were taken before each test to assist in video analysis: an "empty" video containing a short recording of the empty arena, and a calibration video containing a short recording of the arena with specific positions marked. The "empty" video was used in aligning all images of the test recording. The calibration video was used in defining coordinates of regions of interest and converted pixels to millimeters. Subsequent subsections provide additional details of experimental design for each test.

Open Field Test

The open field (58cm W × 58cm D × 58cm H; see Fig. 1B) was constructed from expanded, white PVC (Grainger Industrial Supply). Lighting in the room was set so that the center of the open field measured 300 +/− 5 lux. A calibration video was taken using a poster board on the bottom of the arena with the center and each corner (1 inch from both edges) marked by circles drawn with black marker. Subjects were initially placed in the center of the arena facing away from the movable cart. The video recording software was started and the subject was allowed to freely explore the arena. After 30 minutes of uninterrupted exploration, the subject was returned to its home cage and the number of fecal boli in the arena was counted, a commonly used readout of anxiety originally validated in the rat (Hall 1934).

Light/Dark Test

A mouse-sized place-preference chamber (68.6cm W × 20.3cm D × 38.1cm H; San Diego Instruments; Fig. 1C) was used for a light/dark test. One half of the arena had a black floor and was exteriorly covered with black static cling film. The opposite chamber had a white floor and was interiorly covered with white static cling film (except for the lid to allow video observation). The chamber divider was positioned 4 cm above the floor to allow free access between both chambers. Lighting was set so that the center of the light chamber measured 300 +/− 5 lux. A calibration video was taken using a separate floor panel placed on top of the floor in the white chamber with the center and each corner (1.27 cm from both edges) marked by circles drawn with black marker. Subjects were initially placed in the center of the light chamber facing the entrance to the dark chamber. The video recording software was started and the subject was allowed to freely explore the arena. After 30 minutes of uninterrupted exploration, the subject was returned to its home cage and the number of fecal boli in each chamber was counted.

Predator Cue Test

A y-maze (San Diego Instruments; each arm 10.16 cm W × 55.88 cm D × 50.8 cm H; Fig. 1D) was used for a predator cue test. Lighting was set so that the center of the y-maze measured 300 +/− 5 lux. Two arms were designated as cue arms where either a predator or control cue would be placed. The third arm was designated as the "blank arm". Due to the lighting arrangement of the room, the blank arm was darker (~150 lux) than the center or cue-containing arms. A calibration video was taken using tightly balled black garbage bags inside the ports of each cue arm and a poster board in the blank arm marking the center and edge of the blank arm with black marker. Subjects were initially placed in the center of the empty y-maze facing the blank arm. The video recording software was started and the subject was allowed to freely explore the arena. After 10 minutes, the subject was corralled to the end of the blank arm and a barrier was inserted isolating the subject. Fecal boli were then counted and removed. A cotton ball soaked in 4 ml of red fox urine (Minnesota Traplines) was placed in the port of a randomly chosen cue arm as a predator-associated cue. The fox urine came from a 3.785-liter container with pooled samples from an unknown number of individuals. A cotton ball soaked in 4 ml of white-tailed deer urine (Code Blue) was placed in the port of the other cue arm as a non-predator-associated cue. The doe urine came from one of seven separate individuals. The barrier was then removed and the video recording software was started again. After 20 minutes, the subject was corralled to the end of the blank arm and contained using an insertable barrier. The subject was then anaesthetized using a cassette stuffed with isoflurane-soaked gauze and transferred to a glass jar along with the cassette for blood collection. The cotton balls were removed from the y-maze and fecal boli were counted. After cleaning the y-maze, the cagemate was then tested using the same procedure and cotton balls. Each urine cue was stored at room temperature and concealed from light in rooms separate from the mouse colony and testing room. During the predator cue test, cotton balls soaked in each urine were kept in

empty glass microscope slide boxes before being placed in the testing chamber. Experiments were conducted within seven months after the urine arrived in the lab.

Analysis of Videos

To minimize observer bias, the processing of behavioral videos was blinded with respect to strain. Videos were translated into x and y coordinates of the subject for each frame of the video using the following pipeline. First, the raw videos were converted using ffmpeg via the following command:

```
ffmpeg -i inputFileName.avi -pix_fmt nv12 -f avi -vcodec rawvideo convertedFileName.avi
```

The converted video file was run through an ImageJ script associated with Mousemove (Samson *et al.* 2015), which we modified to label frames deleted due to inability to maintain the framerate or frames when no mouse was detected. Deleted frames and frames when multiple objects were detected were reincorporated using the position of flanking frames. Calibration videos were run through the same process to obtain positions of known locations in the arena. A small subset of videos had a large number of frames (>1%) deleted by erroneously tracking multiple objects. The trajectory files for these videos were manually edited to remove objects that never moved (i.e., not the mouse).

Combined trajectory files were then analyzed to measure a variety of different traits depending on the test (see below). Pixels were translated to mm using known distances between objects in the calibration trajectory file. A movement threshold was set for each subject using the method of Shoji (2016). Output for each trait was recorded for every minute of the test to observe temporal patterns during the test.

The center of the open field was defined as a circle with diameter half the length of a side (29cm). Distance traveled was the sum of all positional changes above the movement threshold in the combined trajectory file.

Times spent past three different thresholds in the light chamber were recorded: 1) 0.5-inch (1.27 cm) from the dark chamber, 2) midpoint of the light chamber, and 3) 0.5 inch (1.27 cm) from the edge of the wall of the light chamber.

For the predator cue test, entrance into each arm was recorded once a mouse was 12 inches (30.48 cm) from the end of the arm. Distance traveled was computed as the sum of all positional changes above the movement threshold in the combined trajectory file.

Corticosterone Quantification

Immediately following the predator cue test, each mouse was anaesthetized using an isoflurane-soaked gauze and transferred to a glass jar. Once unresponsive, the mouse was decapitated, and blood was collected and stored on ice until all tests for the day were completed. Blood was centrifuged at 1200 rpm for 10 minutes at 4°C to collect plasma. Samples were stored at −80°C until assay submission. Plasma corticosterone concentration was quantified by ELISA at the Wisconsin National Primate Research Center.

Statistical Analyses

All statistical analyses were conducted in R (v 3.4.1) (R Development Core Team 2017). Linear models were built separately for each behavior using the lm function {stats}. Behaviors were treated as dependent variables. Strain, sex, age (for the open field test), and cross direction (for F1s) were treated as fixed, independent variables. Interactions suggested by graphical patterns were also evaluated. The

significance of an independent variable was evaluated using additional sum-of-squares tests comparing models that included or excluded the variable. Behaviors in F1s and parental strains were compared using t-tests. F1s from mothers of different strains were tested separately when the cross direction term in the linear model was significant. F1s were inferred to be similar to a parental strain when they were statistically indistinguishable from that strain but distinct from the other parental strain. F1s were inferred to be similar to the mid-parent value, defined as the arithmetic average of the parental means, when they were indistinguishable from that value but distinct from both parental strains. When F1s were statistically distinct from both parental distributions and the mid-parent value, they were inferred to be similar to midpoint values between the mid-parent value and the parental mean to which they were closest.

Data Availability

All computational scripts used in video analysis are available in Supplementary Files 1–9. Raw measurements and associated metadata for each mouse are included in Supplementary Table 1.

Results

Open Field Test

We conducted open field tests at two life stages (juvenile and adult) to investigate how GI mice and mainland mice explore a brightly lit, open environment with high exposure. We observed extensive behavioral differences between GI mice and mainland mice at both juvenile and adult ages (Fig. 2; Table 1). Overall, GI mice spend more time in the center of the arena (Fig. 2A; Table 1), travel more (Fig. 2B; Table 1), and deposit fewer fecal boli than mainland mice (Table 1). Strain differences for time spent in the center of the arena and the number of fecal boli deposited are similar between ages. Alternatively, distance traveled shows a strong strain-by-age interaction, with the disparity between strains expanding after puberty.

F1 offspring from crosses between GI mice and mainland mice show behavioral patterns consistent with a nonadditive genetic architecture with occasional parental effects. For most behaviors, F1 averages are closer to the mainland strain (Fig. 2; Table 2). Only the number of fecal boli deposited by F1 adults is statistically indistinguishable from the mid-parent value (Table 2). Cross direction influences distance traveled in both juveniles and adults. F1s with a GI mother travel less than F1s with a mainland mother (Supplementary Table 2).

Collectively, these results indicate that GI mice evolved an increased willingness to explore novel, risky environments. Whereas boldness-related behaviors are consistent across life stages and cross directions, distance traveled in the open field is influenced by age and parental effects.

Light/Dark Test

To understand how exploration of a novel environment changes when a sheltered area is available, we conducted a light/dark test using a place preference chamber with equally sized light and dark chambers. We found that GI mice spend nearly twice as much time in the light chamber as mainland mice (Fig. 3A; Table 1). GI mice also enter the light chamber nearly twice as many times (Fig. 3B; Table 1). Strain differences are maintained when considering only exploratory bouts past the center of the light chamber (Fig. 3C–D; Table 1). As with the open field test, GI mice deposited less fecal boli, suggesting that baseline anxiety is similar in the two tests (Table 1). These patterns indicate that GI mice evolved increased willingness to leave sheltered areas and to venture farther from shelters compared to mainland mice.

Predator Cue Test

To reveal how exploration is impacted by the presence of a predator cue, we conducted tests in a y-maze with one arm containing a cotton ball soaked in fox urine. In the first 10 minutes of the test with no cues present, both strains show a slight preference for the blank arm where no cue would be placed (Fig. 4A). This pattern could reflect the slight shading of this arm due to lighting constraints in the room. GI mice travel farther overall than mainland mice during these first ten minutes, echoing results from the open field test (Table 1). After both cues are presented, GI mice and mainland mice spend less time in the arm containing the fox urine than in the arm containing the deer urine (Fig. 4A; Table 1). We find no evidence for strain differences in this avoidance of the predator cue.

Plasma corticosterone concentrations collected immediately following the predator cue test do not differ between strains (Fig. 4B, t-test; $P = 0.447$). This result indicates that GI mice are as physiologically stressed as mainland mice by the presence of a predator cue though they continue to deposit fewer fecal boli (t-test; $P < 0.001$). Despite the apparent similarity in anxiety among the two strains, GI mice travel more than mainland mice after cues are presented (Table 1). These findings

suggest that GI mice retain avoidance and stress responses associated with exposure to threats from terrestrial predators even while evolving a greater willingness to explore.

Discussion

Gough Island mice are more exploratory and less fearful than their mainland counterparts in novel, open environments without cues from terrestrial predators. Data from across our experiments demonstrate that GI mice show constant motion throughout each test with little preference for location except when direct cues of terrestrial predators are present. In contrast, mainland mice have high innate anxiety and prefer to remain in regions of each arena characteristic of fearful mice in commonly used behavioral assays (e.g., periphery of the open field, dark chamber of the light/dark box). Since all mice in this study were raised in a common setting, these behavioral differences among inbred strains have a genetic basis.

Although understanding which aspects of the environment on Gough Island stimulated behavioral evolution will require ecological studies, characteristics of GI mice and the island suggest potential causes. GI mice are the largest wild house mice in the world (Gray *et al.* 2015), offering an extreme case of the gigantism commonly observed among island rodents (Foster 1964; Adler and Levins 1994), Larger bodies demand greater energetic requirements, particularly during winter (Peters 1983). The willingness of GI mice to explore could have been driven by expanded caloric demand, especially with a diet that is highly varied and likely opportunistic (Jones *et al.* 2003; Cuthbert *et al.* 2016). Additionally, the evolution of boldness may have facilitated the transition to eating seabird chicks, which are a rich source of nutrients during winter when food is scarce and mortality is high (Cuthbert *et al.* 2016). In contrast to mice inhabiting typical mainland environments, less anxious mice on Gough Island can forage in open areas without danger from predators. For these reasons, exploration and boldness likely provide significant advantages to mice on Gough Island.

Comparisons across juvenile and adult life stages suggest that enhanced exploration and reduced anxiety have evolved along distinct developmental trajectories in GI mice. Although both juvenile and adult GI mice spend more time in the center of an open field and deposit fewer fecal boli, GI mice travel farther than mainland mice only after puberty. Results from other studies support the idea that exploration and boldness can be uncoupled. Pumpkinseed fish approach a novel food source and a potential threat differently (Coleman and Wilson 1998). Wild-caught starlings show greater escape motivation than hand-reared starlings when placed in a new cage, but the two groups of birds respond similarly to novel objects (Feenders *et al.* 2011). Organisms living in a complex environment are expected to benefit from context-specific evolution of behavior (Coleman and Wilson 1998; Sih *et al.* 2004). Exploration, which is tied to the need for resources, including food and shelter, takes on greater importance when mice leave the nest and their mother's care. Alternatively, reducing unnecessary anxiety may increase both juvenile and adult fitness by reducing negative health consequences from continuous stress (Koolhaas *et al.* 1999; Elliot *et al.* 2017). Therefore, natural selection could uncouple the developmental timing of exploration and anxiety.

Rodents living on islands without terrestrial predators are expected to lose the avoidance response to predator cues (Orrock 2010). It is interesting, therefore, that GI mice and mainland mice respond similarly to fox urine. There are multiple explanations for this finding, none of which are mutually exclusive. First, GI mice may not have been on the island long enough for new genetic variants that relax the response to predator cues to arise and spread. Perhaps this phenotype has a smaller mutational target size than general anxiety, a trait for which we observed a heritable reduction in GI mice. A second possibility is that selection on general anxiety may be more direct than selection to remove predator cue responses. The presence or absence of a predator cue response makes no difference when predators are absent, whereas reducing general anxiety can facilitate the optimization of foraging strategies and reduce energy expenditure from unnecessary stress (Elliot *et al.* 2017). A final

explanation is that the pathway for detecting terrestrial predators could play additional functional roles in GI mice. Sulfur-containing byproducts of meat-eating vertebrates are known to be aversive stimuli for rodents (Nolte *et al.* 1994), but there may be other sulfur-containing compounds on Gough Island that mice need to detect. Since fish-eating seabirds are a major source of nutrition for GI mice during the winter season (Cuthbert *et al.* 2016; Caravaggi *et al.* 2019), the ability to locate nests emitting sulfur signals could be beneficial.

It is worth noting that GI mice continue to deposit fewer fecal boli after exposure to predator urine, despite showing similar corticosterone levels to mainland mice following the test. While the number of fecal boli deposited during a behavioral test is a commonly used proxy for anxiety (Flint *et al.* 1995), it is likely influenced by additional factors (e.g., metabolism) and may detect different aspects of anxiety than corticosterone. It is also possible that the method of anesthesia and euthanasia we used prior to plasma collection caused similar levels of anxiety in both mouse strains whereas fecal boli counts represent stress levels during the test itself. Additional measurements of corticosterone concentration both before the predator cue exposure and in individuals that do not undergo behavioral testing will provide a better understanding of the dynamics of physiological stress in these strains.

The phenotypic patterns we documented in F1s provide insights into the genetic architecture that underlies the evolution of new behaviors in GI mice. We found that F1s resemble mainland mice more than GI mice in open field tests, implying that GI mouse alleles are recessive to mainland mouse alleles for several behaviors connected to boldness and exploration. Recessive mutations tend to reduce function (Simmons and Crow 1977), suggesting that the variants of interest could have decreased the expression of genes or pathways that are typically active in mainland mice. The evolutionary trajectory of recessive mutations in GI mice would have depended on their initial frequencies. Whereas new recessive variants would have required stronger selection to become established and spread to high frequency than new dominant variants (Haldane 1927), the probability of fixation of standing variants

that contribute to adaptation is independent of dominance (Orr and Betancourt 2001). The inference of recessive gene action also raises the prospect that behavioral evolution was accomplished through a small number of mutations with large phenotypic effects. Although loci that affect behavior in laboratory strains of mice have been mapped to every chromosome (Flint 2003), alleles with substantial effects exist for some behaviors, including boldness and exploration (Cohen *et al.* 2001; Parker *et al.* 2013).

Our characterization of F1s uncovered additional factors that shape behavior in GI mice. An apparent maternal effect on distance traveled in the open field acts in the opposite direction of the strain effect (i.e., GI mouse mothers reduce distance traveled by F1 offspring). Though this difference could also be caused by a mitochondrial genetic effect, a maternal effect would imply that the genetic increase in distance traveled in GI mice is greater than it appears based on comparisons to mainland mice since this maternal effect must be overcome. Antagonism between maternal and direct genetic effects provides a barrier to selection and could draw populations away from optimal trait values, depending on the degree of covariance between these effects (Cheverud and Moore 1994).

Several caveats accompany our interpretations. The mainland strain we profiled was chosen based on its availability and common usage in mouse genetics. If the behavior of this wild-derived inbred strain departs significantly from the mainland mice from which GI mice are descended, our conclusions about the evolution of new behaviors in GI mice could be incorrect. Western Europe is the most likely ancestral origin of M. m. domesticus in North America and on Gough Island (Gray *et al.* 2014). Detailed reconstruction of behavioral evolution in GI mice will ultimately require behavioral studies in specific source populations, which remain to be identified. Our study also assumes behavior in the laboratory is representative of behavior in the wild. While the simplicity of our experimental design facilitates the identification of causal factors in behavioral differences, those interpretations are limited to the environment in which the tests were conducted. Mice in the wild experience complex

environments without ad libitum access to food and exposed to many context-dependent signals of danger. Other studies have incorporated predator cues and alarm signals as part of the open field test to mimic these natural conditions (Sievert *et al.* 2020). Should these conditions have been present in our open field test, we may have obtained different results. Incorporating more realistic scenarios (e.g., fasting, outside enclosures) in future behavioral studies will help broaden our interpretations of the behavioral changes in GI mice.

Our study is among the first to report inherited differences in behavior between island and mainland populations (Brodin *et al.* 2013; Jolly *et al.* 2018). Island deer mice that evolved larger bodies are less aggressive than their mainland relatives, but this behavioral difference is not heritable (Baier and Hoekstra 2019). Perhaps the extreme ecological conditions on Gough Island (e.g., lack of predators, lack of human commensals, presence of seabird chicks as a source of food in winter) have created particularly strong selective pressure for behavioral evolution. Regardless of the environmental causes, our demonstration of heritable differences between island and mainland populations in a genetic model organism sets the stage for identifying genes responsible for behavioral evolution associated with island colonization. GI mice could also serve as a useful experimental model for exploration and anxiety in humans (Sokolowska and Hovatta 2013; Ashbrook *et al.* 2015) where the list of candidate genes for these behaviors continues to grow (Meier and Deckert 2019).

Significance

Organisms on islands are known to behave differently from mainland organisms. An absence of predators and a different set of natural resources are expected to make island organisms less anxious and more exploratory. We raised two groups of house mice, one from Gough Island in the South Atlantic and one from the mainland Eastern United States, in the same laboratory environment to see if behavioral differences between the two groups are heritable. Mice from both groups were placed in novel enclosures that are known to cause anxiety in rodents. We found that mice from the island are bolder and more exploratory in these enclosures but avoid predator odors in the same way as mainland mice. Our results show that boldness and exploration can evolve after island colonization.

Figure 1.1. Schematic of testing design and arenas. A) Timeline of a subject mouse's life. B) Schematic of the open field arena. The center defined during video analysis is outlined by the dashed circle. C) Schematic of the light/dark box. The subject has free access to both equally-sized chambers during the test. Distance to the center threshold is noted by the dashed line. D) Schematic of the y-maze used in the predator cue test. Each arm is equally sized and freely accessible. The threshold for each arm defined during video analysis is indicated in the right arm by the dashed line. Locations of the predator and control cues are noted with black dots.

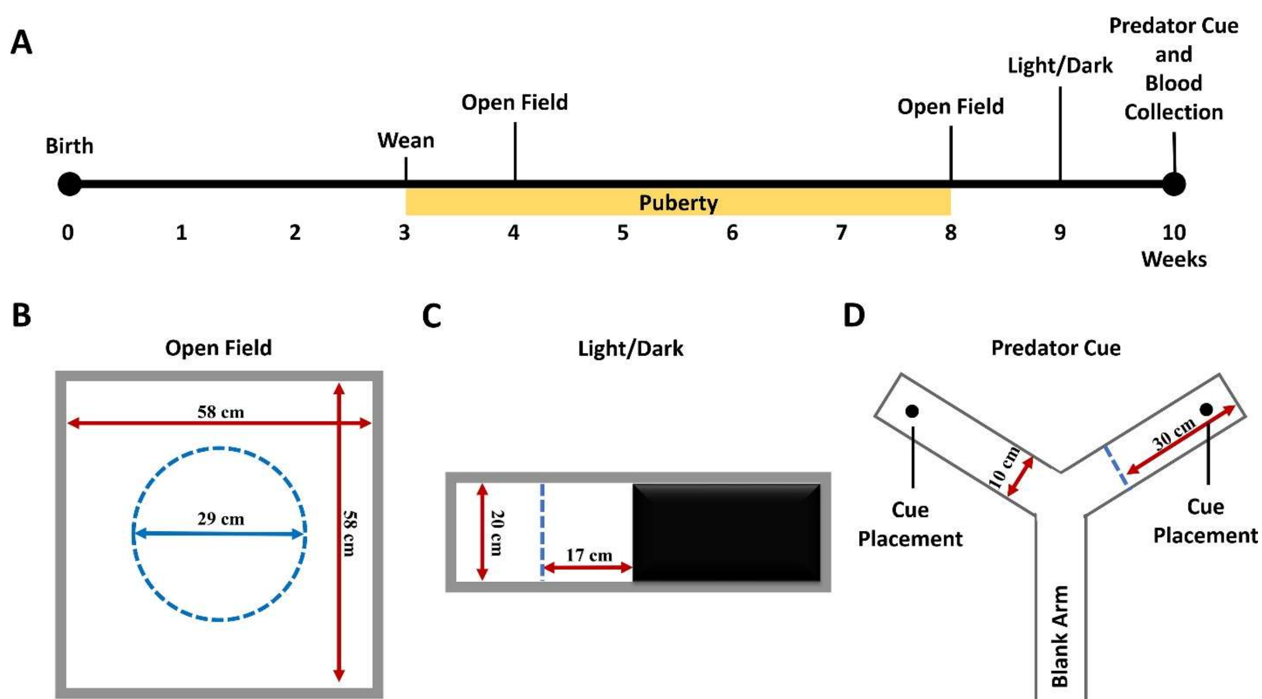

Figure 1.2. Open field comparisons between strain and ages. Means across sexes are designated by horizontal black bars. F1s are separated based on the strain of the mother. A) Time spent in the center of the open field. B) Distance traveled in meters.

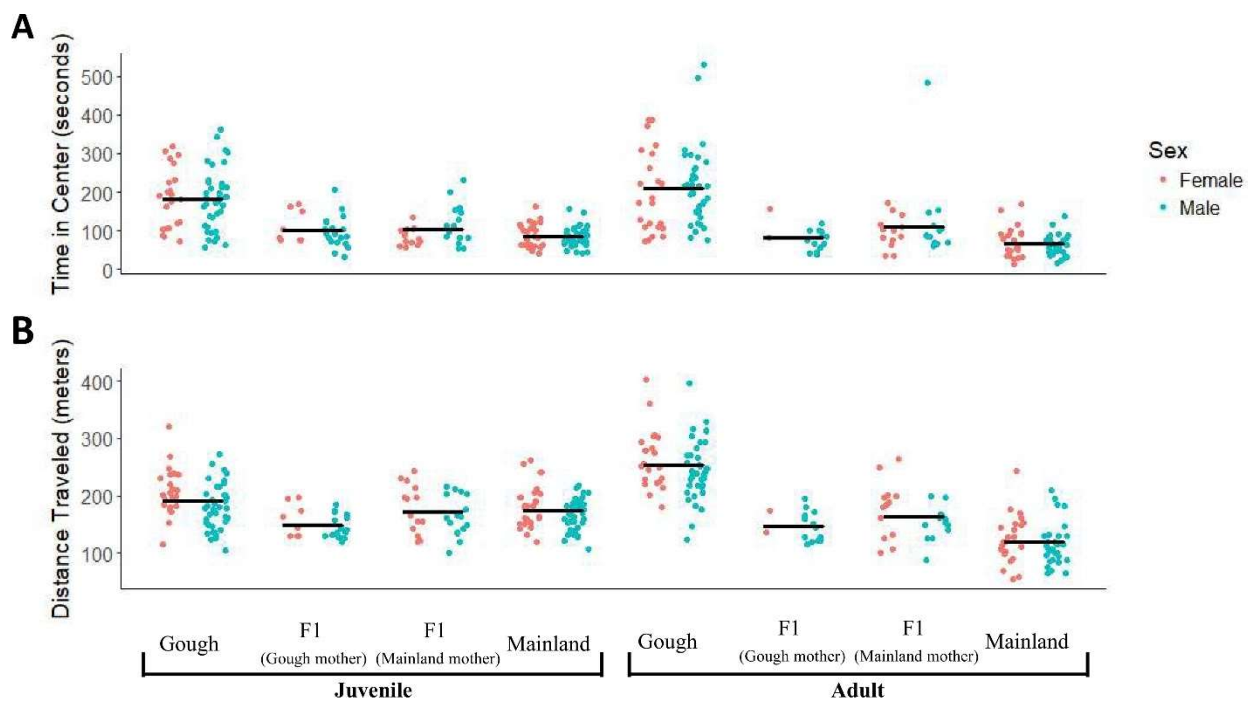

Figure 1.3. Results from light/dark test. Means across sexes are designated by horizontal black bars. A) Left: amount of time spent in light chamber of light/dark box. Right: number of entrances to the light chamber. B) Left: amount of time spent past the center of the light chamber. Right: number of crosses past the center of the light chamber.

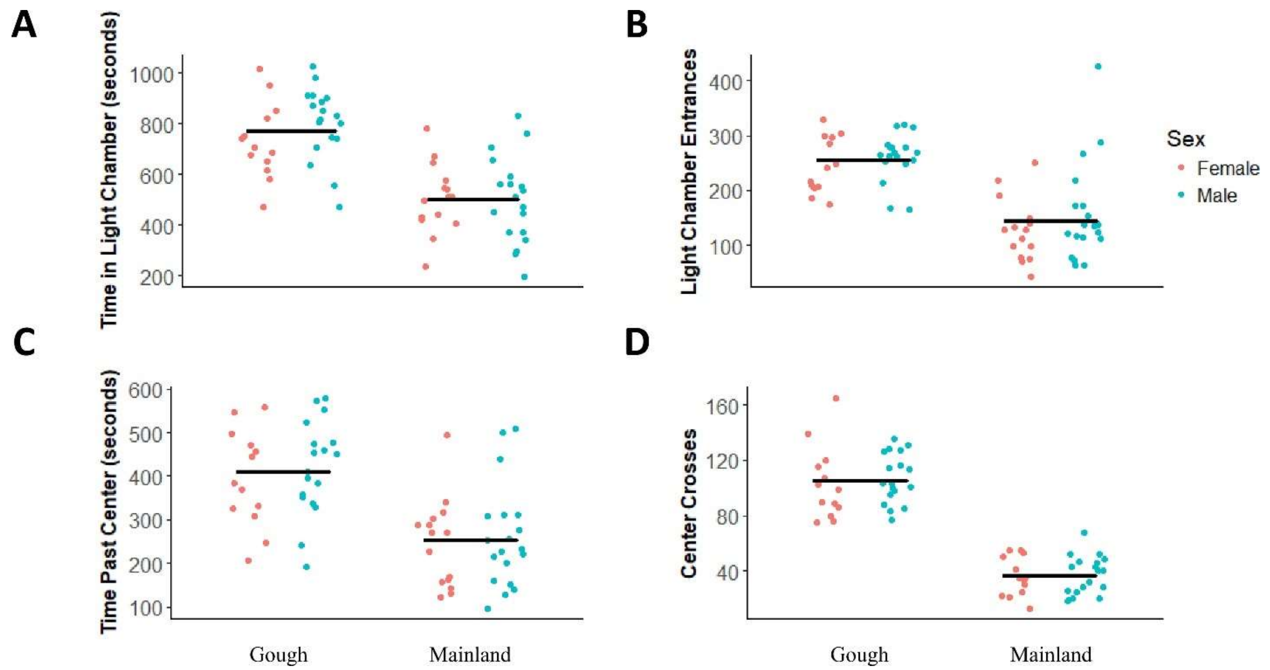

Figure 1.4. Results from predator cue test. A) Mean ratio and standard error of time spent in arm with a given cue to time spent in blank arm. Ratios on the left are for the ten minutes of exploration before cues were added. B) Corticosterone concentration of mice immediately after the predator cue test. Means across sexes are designated by horizontal bars. Note the assay has a maximum detection limit of 1000 ng/mL.

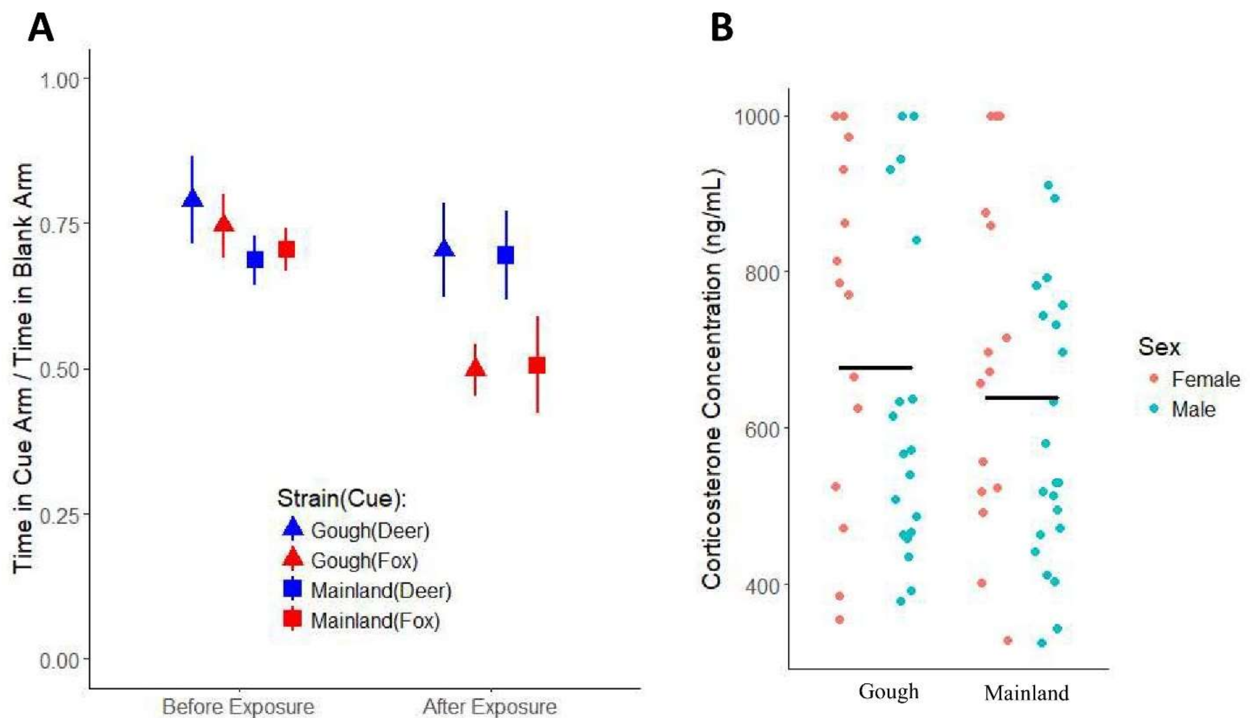

Table 1.1. Summaries of linear model results for each trait across experiments. The third column shows transformations performed on the dependent variable. The fourth column shows significant independent variables among Sex, Strain, and Age (for traits from the open field test). When none of these predictors were significant, the overall mean is presented as the Intercept Estimate.

Experiment	Trait	Transformation	Independent Variables	Intercept Estimate	Strain (Mainland) Effect	Strain p-value
Open Field	Distance Traveled (meters)	None	Sex, Strain, Age, Strain*Age	201.686	−17.416	0.02
	Time Spent Mobile (seconds)	None	Sex, Strain, Age, Strain*Age	648.324	−6.506	0.749
	Time in Center (seconds)	Natural Log	Strain	5.16728	−0.91655	<0.001
	Number of Fecal Boli	None	Strain	6.833	2.7137	<0.001
Light/ Dark Box	Time in Light Chamber (seconds)	None	Strain	774.53	−271.62	<0.001
	Light Chamber Entrances	Natural Log	Strain	5.52242	−0.678	<0.001
	Time Past Center (seconds)	None	Strain	409.12	−155.35	<0.001
	Center Crosses	Natural Log	Strain	4.63887	−1.10467	<0.001
	Number of Fecal Boli	None	Strain	6.9697	2.5829	<0.001

Predator Cue	Ratio of Time in Cue Arm/Blank Arm Pre-exposure	None	None	0.723555	-	-
	Ratio of Time in Control Arm/Blank Arm Pre-exposure	None	None	0.734002	-	-
	Distance Traveled (meters) Pre-exposure	None	Strain	80.37	−28.213	<0.001
	Time Spent Mobile (seconds) Pre-exposure	None	Strain	186.497	−40.137	<0.001
	Ratio of Time in Cue Arm/Blank Arm Post-exposure	None	None	0.501996	-	-
	Ratio of Time in Control Arm/Blank Arm Post-exposure	None	None	0.698775	-	-
	Distance Traveled (meters) Post-exposure	None	Strain, Sex	131.017	−35.684	<0.001
	Time Spent Mobile (seconds) Post-exposure	Natural Log	Strain, Sex	5.86979	−0.14411	<0.001
	Corticosterone Concentration (ng/mL)	None	Sex	715.63	-	-

Table 1.1 continued. Summaries of linear model results for each trait across experiments. The third column shows transformations performed on the dependent variable. The fourth column shows significant independent variables among Sex, Strain, and Age (for traits from the open field test). When none of these predictors were significant, the overall mean is presented as the Intercept Estimate.

Experiment	Trait	Sex (Male) Effect	Sex p-value	Age (Adult) Effect	Age p-value	Strain:Age Effect	Strain:Age p-value
Open Field	Distance Traveled (meters)	−17.896	0.001	62.007	<0.001	−116.698	<0.001
	Time Spent Mobile (seconds)	−44.306	0.004	99.081	<0.001	−245.43	<0.001
	Time in Center (seconds)	-	-	-	-	-	-
	Number of Fecal Boli	-	-	-	-	-	-
Light/ Dark Box	Time in Light Chamber (seconds)	-	-	-	-	-	-
	Light Chamber Entrances	-	-	-	-	-	-
	Time Past Center (seconds)	-	-	-	-	-	-
	Center Crosses	-	-	-	-	-	-
	Number of Fecal Boli	-	-	-	-	-	-

Predator Cue	Ratio of Time in Cue Arm/Blank Arm Pre-exposure	-	-	-	-	-	-
	Ratio of Time in Control Arm/Blank Arm Pre-exposure	-	-	-	-	-	-
	Distance Traveled (meters) Pre-exposure	-	-	-	-	-	-
	Time Spent Mobile (seconds) Pre-exposure	-	-	-	-	-	-
	Ratio of Time in Cue Arm/Blank Arm Post-exposure	-	-	-	-	-	-
	Ratio of Time in Control Arm/Blank Arm Post-exposure	-	-	-	-	-	-
	Distance Traveled (meters) Post-exposure	−17.732	0.004	-	-	-	-
	Time Spent Mobile (seconds) Post-exposure	−0.1002	0.01	-	-	-	-
	Corticosterone Concentration (ng/mL)	−100.01	0.048	-	-	-	-

Table 1.2. Summary of effects influencing behaviors in the open field. A "+" indicates significance of that variable for the given trait. F1 groupings are based on which values (mainland mean, GI mean, or midparent) the F1 distribution is significantly different from using a t-test. For traits with a significant Cross Direction effect the two groups were treated separately.

Experiment	Trait	Sex Effect	Cross Direction Effect	F1 Grouping
Open Field Juvenile	Distance Traveled	+	+	Underdominant (GI Mother), Mainland (Mainland Mother)
	Time in Center	-	-	Midparent/Mainland midpoint
	Number of Fecal Boli	-	-	Gough Island
Open Field Adult	Distance Traveled	+	+	Midparent/Mainland midpoint
	Time in Center	-	-	Midparent/Mainland midpoint
	Number of Fecal Boli	-	-	Midparent

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Chapter 2

Genetics of behavioral evolution in giant mice from Gough Island

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http://doi.org/10.1098/rspb.2022.2603

Contributions were as follows: JAS helped design the study, carried out the behavioral testing, performed the video analysis, conducted the statistical testing, and drafted the manuscript; MJN helped design the study, and critically revised the manuscript; BAP helped design the study, and critically revised the manuscript.

Abstract

The evolution of behavior on islands is a pervasive phenomenon that contributed to Darwin's theory of natural selection. Island populations frequently show increased boldness and exploration compared to their mainland counterparts. Despite the generality of this pattern, the genetic basis of island-associated behaviors remains a mystery. To address this gap in knowledge, we genetically dissected behavior in 613 F2s generated by crossing inbred mouse strains from Gough Island (where they live without predators or human commensals) and a mainland conspecific. We used open field and light/dark box tests to measure seven behaviors related to boldness and exploration in juveniles and adults. Across all assays, we identified a total of 41 quantitative trait loci (QTL) influencing boldness and exploration. QTL have moderate effects and are often unique to specific behaviors or ages. Function-valued trait mapping revealed changes in estimated effects of QTL during assays, providing a rare dynamic window into the genetics of behavior often missed by standard approaches. The genomic locations of QTL are distinct from those found in laboratory strains of mice, indicating different genetic paths to the evolution of similar behaviors. We combine our mapping results with extensive phenotypic and genetic information available for laboratory mice to nominate candidate genes for the evolution of behavior on islands.

Introduction

Organisms on islands behave differently than their mainland counterparts (Darwin 1840; Cooper *et al.* 2014; Gavriilidi *et al.* 2022). Island environments present novel resources, predation pressures, and competition (Williamson 1981; Grant 1998), leading to the evolution of new foraging strategies and activity patterns in island colonizers (Creel and Christianson 2008; Elliot *et al.* 2017). Investigating the genetic basis of behavioral evolution in island populations has the potential to reveal mechanisms by which organisms respond to environmental change.

The behavior of insular populations has long been of interest to evolutionary biologists. Darwin first used the term "island tameness" (Darwin 1840) to describe the reduction in anti-predator behaviors commonly shown in insular organisms (Cooper *et al.* 2014; Gavriilidi *et al.* 2022; Blumstein and Dnaiel 2005). As part of the "island syndrome" (Adler and Levins 1994), insular populations show reduced aggression compared to mainland conspecifics (Halpin 1981; Gray and Hurst 1998; Baier and Hoekstra 2019). The ecological conditions that drive novel behaviors in insular populations, such as island area, island remoteness, and predator prevalence, have been subjects of many investigations (Ganem 1998; Orrock 2010; Juette *et al.* 2020; Blanco *et al.* 2014; Raia *et al.* 2010). However, few studies have shown a heritable component to island-associated behaviors (Brodin *et al.* 2013; Jolly *et al.* 2018), a requirement for evolution and the identification of causative loci.

A striking example of an insular population with documented, heritable changes in behavior is the house mice that invaded Gough Island (Stratton *et al.* 2021), a remote island in the middle of the South Atlantic. These mice belong to the subspecies *Mus musculus domesticus* (Gray *et al.* 2014) and colonized the remote island probably via sealing vessels from Western Europe (Verrill 1895; Wace 1961) a few hundred to a few thousand generations ago (Rowe-Rowe and Crafford 1992; Payseur and Jing

2020). Since colonization of Gough Island (hereafter "GI"), the mice evolved an exceptionally large size (Jones *et al.* 2003; Gray *et al.* 2015), a pervasive expansion of the skeleton (Parmenter *et al.* 2016), a stronger bite (Parmenter *et al.* 2020), and a new mandible shape (Parmenter *et al.* 2022). These unusual phenotypes echo the unusual ecology of the island where the mice live without predators or human commensals (Rowe-Rowe and Crafford 1992) and feed primarily on invertebrates and seeds (Jones *et al.* 2003). In the winter, GI mice predate on seabird populations consuming an estimated 2 million chicks and/or eggs per year (Caravaggi *et al.* 2019). Following expectations for island colonizers and corresponding to their novel predatory behavior, GI mice are bolder and more exploratory in novel environments compared to mainland conspecifics (Stratton *et al.* 2021). A genetic investigation of these behavioral changes promises to provide insight into long-standing questions about behavioral evolution.

In addition to being important parameters for fitness, behaviors associated with island adaptation, including reduced anxiety, are related to those underlying human behavioral disorders (Stein and Bouwer 1997; Hohoff 2009). Classical inbred strains of house mice are established models for human behavior (Fisch 2009) and are routinely used to study its genetic architecture (Flint *et al.* 1995; Wehner *et al.* 2001; Flint 2003). Cross-species comparisons have successfully identified candidate genes underlying behavior in both mice and humans (Hovatta and Barlow 2008; Sokolowska and Hovatta 2013; Ashbrook *et al.* 2015). Despite this wealth of knowledge from laboratory strains of house mice and the expectation that natural populations will provide new insights into the inheritance of complex traits (Gasch *et al.* 2016), genetic examinations of behavioral evolution in wild mice remain rare. In this study, we use GI mice as a model system to characterize the genetic architecture of the evolution of boldness and exploration on islands.

Methods

Mouse Strains and Husbandry

To generate F2 mice, we conducted intercrosses between inbred strains of GI mice (produced in our laboratory) and mainland mice, the same strains used by Stratton *et al*. (2021). In 2009, GI mice were live-caught and shipped to the University of Wisconsin School of Veterinary Medicine Charmany Instructional Facility, where a breeding colony was established (Gray *et al.* 2015). GI mice were then bred for over 20 generations of brother-sister mating to establish an inbred line. Mainland mice belong to the WSB/EiJ inbred strain (JAX stock #001145) founded from breeding pairs caught in Maryland and were maintained in the same colony as GI mice for the same period of time. GI mice and house mice from the eastern coast of North America are likely descended from Western European populations (Gray *et al.* 2014; Agwamba and Nachman 2022), though the geographic locations of specific source populations are unknown. Husbandry and weighing protocols were identical to those applied by Stratton *et al*. (2021) (see Supplementary File 1). F1s were generated by crossing mice from the GI strain and the mainland strain in both maternal directions (Figure 1D). F1 siblings were crossed to each other to generate F2s.

Behavioral Assays

All behavioral assays were conducted in a similar manner to Stratton *et al*. (2021), restated in Supplementary File 1 with differences noted. Phenotyping was conducted from January 2020 to July 2021. Each subject was tested three times with the following regimen (Figure 1A): open field (4 and 8 weeks old +/- 1 day; Figure 1B), and light/dark box (9 weeks old +/- 1 day; Figure 1C). Videos were recorded for each test (for 30 minutes) and run through a modified ImageJ script associated with

Mousemove (Samson *et al.* 2015) to create trajectory files with x and y coordinates of the mouse at each frame. To conduct function-valued trait analyses, behavioral measurements were extracted from trajectory files at every minute of the test using an independent pipeline (see Supplementary File 1). After each assay, the number of fecal boli in the arena was counted ("Fecal Boli"). For "Time in Center", the center of the open field was defined as a circle with a 26.59 cm radius (14.5 cm radius in Stratton *et al.* (2021)) which stretches to 1 inch from the edge of the arena. "Distance Traveled" was the sum of all positional changes above the movement threshold determined by the method of Shoji *et al.* (2016).

Time spent past two different thresholds in the light chamber were recorded: 1) 1.27 cm (0.5-inch) from the dark chamber, ("Time in Light Chamber") and 2) midpoint of the light chamber ("Time past Center"). The number of crosses past these thresholds was also recorded ("Light Chamber Entrances" and "Center Crosses" respectively).

Genotyping

Liver tissue was collected from each F2 and submitted to Neogen for genotyping on the Giga Mouse Universal Genotyping Array (https://www.neogen.com/categories/genotyping-arrays/gigamuga/, see Supplementary File 1). Previous versions of this array were used successfully to map QTL for morphological traits between GI mice and WSB/EiJ mice (Gray *et al.* 2015; Parmenter *et al.* 2016; Parmenter *et al.* 2022). After quality control checks (see Supplementary File 1), a final dataset of 613 F2s with genotype information at 31,681 informative markers was used for QTL analyses.

QTL Analyses

All statistical analyses were conducted in R (v 3.4.1; v 4.1.3 for function-valued trait analyses and

scans for epistasis) (R Development Core Team 2019). Two approaches were taken to identify QTL for behavior (see Supplementary File 1): 1) single trait analyses using the value of each phenotype at the end of the behavioral assay, and 2) function-valued trait analyses combining information across the entire behavioral assay.

Single Trait Mapping

Single trait (hereafter "ST") analyses were conducted in R/qtl (Broman and Sen 2009) using the value of each behavioral trait from the culmination of the assay. Analyses used Haley – Knott regression (Haley and Knott 1992) with covariates listed in Supplementary Table 1. To determine which covariates to include in the QTL scan, environmental and pedigree-related predictors were evaluated using linear models for each trait. Independent variables were included as additive covariates when deemed significant by an additional sum-of-squares test comparing models that included or excluded the variable. To search for QTL-by-sex interactions, we conducted scans with sex as an interactive covariate for traits where sex was included as an additive covariate (Time in Center Juvenile, Distance Traveled Juvenile, Distance Traveled Adult, Fecal Boli Adult, Light Chamber Entrances, Center Crosses, and Fecal Boli Total).

Function-valued Trait Mapping

To incorporate temporal dynamics of behaviors during the course of the assay, we conducted function-valued trait QTL mapping as implemented in r.funqtl (Kwak *et al.* 2015). Fecal boli were counted only at the end of each assay and were not included in this analysis. Values for each behavior were calculated at each minute of the test (1-30) and the collection of these values was converted to a

set of functional principal components (fPCs). The fPCs were then fit to the same covariates used in ST analyses and residuals were extracted for QTL mapping. Three different mapping approaches were used. "HK" multivariate mapping follows the method of Knott and Haley (2000). "SL" and "ML" mapping scan each fPC individually and extract the average and maximum LOD scores, respectively. These three approaches are designed to capture QTL with a wide range of effect sizes and durations of activity.

Scans for Epistasis

We applied two methods to search for epistatic QTL (see Supplementary File 1). First, we conducted scans for pairs of interacting QTL using the function scantwo() in R/qtl (Broman and Sen 2009). All pairs of autosomal locations were considered. Second, we searched for QTL with differences in variance among genotypes using the function scaneonevar() in R/qtl.

Candidate Gene Nomination

Candidate genes for each QTL were nominated based on three primary criteria (see Supplementary File 1): 1) associations with phenotypes of interest in mice and humans, 2) sequence differences between the two inbred strains used in the cross, and 3) expression in brain regions of interest in mice.

Evidence for Selection

To examine evidence for positive, directional selection acting on candidate genes in GI mice, we compared our findings to those presented in Payseur and Jing (2020). These authors computed posterior probabilities of directional selection across the genome using 14 wild-caught GI mice and their

mainland counterparts (see Supplementary File 1). We extracted the maximum posterior probability within each candidate gene region to evaluate evidence for selection acting on behavioral candidate genes.

Data Availability

All computational scripts used in video analysis are available in Supplementary Files 3-9. Raw measurements and associated metadata for each F2 used in QTL analyses are included in Supplementary Table 2.

Results

Phenotypic Variation

Stratton *et al*. (2021) demonstrated that GI mice and mainland mice show extensive behavioral differences across assays (Figure 2, green and blue bars). GI mice spend more time in the center of the open field, deposit fewer fecal boli, and explore the light chamber more often than mainland mice. GI mice travel more in the open field than mainland mice, but primarily as adults. All behavioral traits show approximately normal distributions across F2 mice, except for distance traveled as adults (which is right-skewed) (Figure 2). Means of F2 distributions are often shifted away from the midparent value (the arithmetic average of parental strain means), with the direction of this shift varying among traits. F2 means resemble GI mice for counts of fecal boli (across all assays) and for the number of light chamber entrances. F2 means and mainland mouse means are more similar for time spent in the center of the open field and time spent past the center of the light/dark box.

Correlations among Traits

To examine relationships among phenotypes, we calculated pairwise correlations between all traits across F2s (Figure 3). The same traits measured in juveniles and adults using the open field test are positively correlated. The number of center crosses in the light/dark box is positively correlated with distance traveled in the open field at both juvenile (r=0.425, Pearson's product-moment correlation; $P <$ 0.001) and adult (r=0.568; $P < 0.001$) stages, suggesting these traits capture the same underlying behavior. Body size is weakly negatively correlated with distance traveled at both ages (juvenile: r=-0.182; $P < 0.001$; adult: r=-0.151; $P < 0.001$) and with the number of center crosses in the light/dark box (r=-0.219; $P < 0.001$), but size is uncorrelated with fecal boli counts in the open field test ($P > 0.05$). Notably, different behaviors within the open field test have correlations near zero. Fecal boli counts in

the light/dark box are also uncorrelated with other behaviors in the light/dark box. Together, these results cluster behaviors into three groups: 1) anxiety, captured by time in the center of the open field and time in the light chamber of the light/dark box, 2) exploration, captured by distance traveled in the open field and center crosses in the light/dark box, and 3) defecation, measured by fecal boli counts. Correlations between traits within an assay decrease across timepoints (Supplementary Figure 1). Such declines are expected when the underlying functions for each trait are approximately linear with time and with different slopes. Overall, these findings suggest that the suite of behaviors we quantified are genetically separable and context dependent.

QTL for Behavioral Evolution

We identified a total of 41 QTL across ages, traits, and analyses (Table 1, Figure 4). We uncovered no evidence for epistatic QTL. Our experimental design provides an unusual opportunity to compare the genetics of the same behavior at two developmental stages. Although common QTL sometimes affect the same trait measured in juveniles and adults, genetic architectures differ at the two ages. For example, whereas more QTL modulate distance traveled and the number of fecal boli in adults, more QTL regulate time in center in juveniles.

Different traits measured at the same age also show distinct genetic architectures (Table 1). In juveniles, time in center, distance traveled, and number of fecal boli have no QTL in common. Although QTL confidence intervals on chromosomes 2, 4, 8, and 14 overlap for open field traits quantified in adults, the best estimated positions of these QTL are far apart and confidence intervals are challenging to estimate, especially for methods that incorporate information across timepoints during the experiment. The QTL on chromosome 19 is unique to distance traveled in the open field. The QTL on chromosome 7 is unique to exploration in adults. Most QTL are distinct across behavioral traits measured within the juvenile open field test and light-dark box test. The QTL on chromosome 1 is

associated with both sexes in juveniles but with females in adults for the light/dark box. Our application of four statistical approaches to QTL mapping (see "Single Trait Mapping" and "Function-valued Trait Mapping" in "Methods") expanded the variety of QTL we discovered (Table 1). Although many QTL were identified by all four methods, each method detected at least one unique QTL and some QTL were supported by two or three methods. Variation among QTL across methods likely reflects differences in the ability to capture the underlying behavior. Function-valued trait mapping and single trait mapping operated on different data for the same trait, with the former incorporating measurements from every minute of an assay and the latter restricted to cumulative, end-point measurements. Overall, most QTL appear to be context-specific, detected only at specific ages, in certain assays, or by individual methods.

Additive Effects of QTL

One characteristic shared across QTL is their moderate effect size. Additive effects are typically less than 0.4 phenotypic standard deviations (Figure 5; Table 2). However, these effects are associated with noticeable shifts in observed behaviors (Supplementary Figure 2). Strikingly, the magnitude of the additive effect of a QTL often changes across timepoints within an assay, with some QTL showing peak effects at early timepoints and others increasing in magnitude over time (Figure 5A and B). GI mouse alleles at the QTL on chromosome 3 in juveniles decrease the amount of time spent in the open field by approximately 10 seconds after 15 minutes of exploration, but do not cause any additional effects for the remainder of the assay (Supplementary Figure 2A). In contrast, time spent in the light chamber increases by approximately 0.5 seconds per minute for each GI mouse allele at the two QTL on chromosomes 5 and 8 (Supplementary Figure 2E). Whether the GI mouse allele increases or decreases the trait value varies across QTL (Figure 5; Supplementary Table 3; Supplementary Figure 2); the direction of additive effects and the direction of the difference in parental means do not consistently match (Table 1). Additive effects at most QTL maintain their sign across the entire assay, including those

for time in center as juveniles, a trait for which the difference in parental means changes during the assay (Table 1; Supplementary Figure 3A). The additive effect of the QTL on chromosome 9 shifts in sign during the assay, though the pattern is reversed between distance traveled in the open field and time past center in the light/dark box (Table 1; Figure 5D and G). The effect of the QTL on chromosome 1 for center crosses in the light/dark box is restricted to females (Figure 6).

Dominance Effects of QTL

Dominance is present across all traits with effects in both directions (Table 1). Dominance is less apparent for QTL associated with distance traveled in the open field (Table 1). Among QTL for anxiety-related traits (time in center, time in light chamber, and fecal boli) with consistent signs of effects across the assay and evidence of dominance, GI mouse alleles are dominant more often than expected by chance (12 out of 15 QTL; P = 0.035; two-tailed cumulative binomial probability). We present qualitative results in Table 1 as dominance effects vary in magnitude across traits and timepoints with substantial standard errors (Supplementary File 2).

Contributions to Strain Differences

Collectively, the additive effects of QTL identified for each trait explain, on average, 44.1% of the difference in means between GI mice and mainland mice at the end of each assay (Supplementary Figure 4; Supplementary File 2). The percent difference explained varies substantially among traits (Supplementary File 2, Supplementary Table 3). In juveniles, QTL for time spent in the center of the open field explain 93.7% of the difference in means between GI mice and mainland mice at the final timepoint, whereas the single QTL for time spent past the center of the light chamber explains only 7.4% of the parental strain difference.

Candidate Genes

We identified strong candidate genes for each QTL (Supplementary Table 4). 2,098 genes (Supplementary Table 5) were pulled down in the initial filtering step for associations to behavior (see Supplementary File 1). Of these 2,098 genes, Supplementary Table 4 highlights 24 genes with relevant sequence differences between GI mice and mainland mice (i.e., protein-coding changes or changes in regions conserved across placental mammals). These genes were chosen based on three criteria: 1) known influences on behaviors of interest in mice and humans, 2) proximity of the gene to the peak position of the QTL, and 3) the ubiquity of expression in three brain regions known to be involved in anxiety and exploration (i.e., the hippocampus, amygdala, and prefrontal cortex).

Evidence for Natural Selection

To explore whether selection contributed to the evolution of behavioral QTL in GI mice, we compared our candidate genes to posterior probabilities of selection calculated in wild populations of GI mice and mainland mice (Payseur and Jing 2020). Three candidate genes overlap 5kb windows with strong signatures of selection (posterior probability >= 0.9): *Dab1, Cadps2,* and *Syn3*. *Lsamp* overlaps a window with a posterior probability of selection of 0.584. None of the sequence differences listed in Supplementary Table 4 (in conserved sites or conferring protein coding changes) fall in these windows with maximum posterior probabilities of selection.

Discussion

We provide a rare glimpse into the genetics underlying island-associated behavioral evolution. Our study draws on the vast resources and knowledge base of laboratory house mice to improve the growing understanding of behavioral evolution (van Oers and Mueller 2010; Niepoth and Bendesky 2020; York 2018; Hoekstra and Robinson 2022). The evolution of striking behavioral differences between GI mice and mainland mice involved multiple genetic changes with individually modest phenotypic effects. The genetic architecture we uncovered emphasizes the importance of context for boldness and exploration, with the action of many loci localizing to certain ages, assays, or timepoints within assays.

Our study highlights advantages of designing experimental assays and statistical analyses to capture dynamic genetic effects underlying transitory behaviors. Extending the period of observation to 30 minutes, a longer interval of free exploration than is typical in open field and light/dark box experiments, enables the measurement of both initial and acclimatized responses to novel environments. Assessing the same mice at two ages yields a developmental perspective on behaviors whose connection to fitness is expected to differ between juveniles and adults. Function-valued trait mapping considers the trajectory of behavior across an assay, a rich description that makes it possible to identify loci with phenotypic effects that change over time.

Correlations among phenotypes across F2s provide insights into the process of behavioral adaptation in GI mice. Behaviors separated into three distinct groups: anxiety, exploration, and defecation. The QTL we detected largely echo this grouping, suggesting evolution proceeded independently within each category. Interestingly, body size is negatively correlated with exploration, a pattern also seen by Zhang and Gershenfeld (2003). Increased body size and enhanced exploration are

expected to be associated with higher fitness in GI mice. In this scenario, the negative correlation between these traits may have created conflict during their evolution. This inference is consistent with the "constraint" hypothesis for the evolution of syndromes described and demonstrated in field crickets (Royautè *et al.* 2020). However, exploration may not be an important determinant of fitness on the island if resources are readily available, allowing body size to evolve more rapidly.

We expand the extensive genetic dissection of house mouse behavior into wild populations. Mapping studies using classical inbred strains of house mice revealed QTL influencing behavior scattered across the genome (Flint 2003). Notably, chromosomes 1, 15, and 18, which show dense clusters of QTL influencing emotionality in the open field test (Willis-Owen and Flint 2006), are underrepresented in our results. This discordance suggests that natural populations and classical inbred strains of mice followed distinct genetic paths to evolve similar behaviors and highlights the potential of wild-derived strains to identify novel candidate genes influencing anxiety and exploration. Still, some of our findings resemble those for laboratory mice. In one example, Turri *et al.* (2001) measured 22 phenotypes across 9 behavioral assays in over 1,600 F2s created by intercrossing the DeFries mouse strain selected for high activity in the open field to the DeFries strain selected for low activity (DeFries *et al.* 1978). Resembling our results, Turri *et al.* (2001) found many QTL that are context-specific with a few QTL that affect multiple behaviors. Furthermore, these authors noted that the QTL with the largest effect on anxiety had no effect on defecation, echoing our findings that suggest these traits are genetically separable. Fecal boli counts might capture other forms of anxiety (called "emotional elimination" by Turri *et al.* (2001)) or be more closely connected to metabolism.

We used multiple lines of evidence to identify strong candidate genes for the evolution of behavior. Several candidate genes are involved in evolutionarily ancient signaling processes in the brain, including the glutamate receptors *Gria2* and *Grm2* (Moroz *et al.* 2021). Other candidate genes, such as

Dab1, *Eya3*, *Mapk10*, *Lsamp*, *Cadps2*, and *Slit1*, function in neuronal development and/or axon guidance (Arimitsu *et al.* 2021; Söker *et al.* 2008; Myers *et al.* 2020; Singh *et al.* 2018; Shinoda *et al.* 2018; Blockus and Chédotal 2016). These two categories of genes, those expressed at the time the behavior is enacted and those involved in development at earlier stages, underscore the question of which biological processes are altered when boldness and/or exploration evolve in nature.

Our findings also shed light on how evolution shapes behavior. The direction of additive effects (i.e., whether the GI mouse allele increases or decreases the trait) varies across QTL. The mixture of QTL effects contributes to the transgressive distribution of behaviors in F2s, where phenotypic ranges exceed the parental means (Figure 2). This pattern argues against directional selection toward a consistent optimum as a primary cause of the full suite of behaviors we examined (Orr 1998). Nevertheless, additive effects of QTL for anxiety-related traits (i.e., time in center of the open field, time in the light chamber) all align with parental differences. This finding supports the proposal that bolder mice are more fit on Gough Island because they spend less time and energy on costly predator avoidance behavior in an environment without predators (Elliot *et al.* 2017). Additionally, increased boldness may enhance novel predation of seabird chicks, an important source of food during the winter (Caravaggi *et al.* 2019; Cuthbert *et al.* 2016). Evolution of these traits may have been accentuated by dominance favoring GI mouse alleles for boldness.

The evolutionary histories of the QTL we identified merit deeper investigation. The genetic changes responsible for QTL could have evolved along either the GI mouse lineage or the mainland mouse lineage. Although the mainland strain we used has been housed in a laboratory environment for several decades, mice from this strain maintain behaviors typical of wild house mice (Schoenrock *et al.* 2016). Comparisons to 62 mouse strains in the Mouse Phenome Database (Kumar 2021) place GI mice in the phenotypic extremes for open field behaviors. As a result, the strains of mice we used should

provide good comparisons for revealing the genetic basis of island-associated behaviors.

Our results lay the groundwork for additional questions that future studies may address. First, as is typical for genetic mapping studies that rely on a single generation of recombination, many QTL we identified have broad confidence intervals and may contain multiple causative mutations. Fine-mapping has revealed some behavioral QTL in laboratory mice to be caused by several closely linked genetic changes (Willis-Owen and Flint 2006; Parker *et al.* 2013). Development and refinement of congenic strains that harbor GI mouse alleles may reveal additional loci with varying effects. Additionally, the relationships between the behaviors we measured and others associated with the island syndrome are unknown. Reduced aggression is commonly associated with island life in rodents (Adler and Levins 1994; Baier and Hoekstra 2019). Though we have not measured aggression directly in GI mice, we suspect from rates of injuries in our colony that it is reduced. Since boldness and aggression are thought to be related (Sih *et al.* 2004), it would be useful to assess the relationship between these traits in GI mice. Lastly, our inbred strain of GI mice is only a partial representation of the genetic diversity of GI mice. Mice from highland and lowland sites on Gough Island are known to differ in both body size and rates of predation on seabird chicks (Cuthbert *et al.* 2016). Additional investigations among genetically distinct GI mice may reveal new insights into the evolution of behavior on islands.

Our study provides an initial genetic portrait of the evolution of boldness and exploration on an island with key environmental differences from the mainland. We hope future studies that dissect the genetic causes of island tameness in other populations will enable comparisons among loci underlying this pervasive phenomenon. Identifying genes and variants responsible for behavioral variation begins with pioneering mapping studies and is a vital part of understanding the evolution of behavior in nature.

Figure 2.1. Summary of behavioral phenotyping timeline and assays. A. Timeline of a subject mouse's life. B. Schematic of the open field arena. The center defined during video analysis is outlined by the dashed circle. C. Schematic of the light/dark box. The subject had free access to both equally sized chambers during the test. Distance to the center threshold is noted by the dashed line. D. Cross design and sample sizes. F1s were generated in both maternal cross directions. F2s were generated through sibling matings of F1s.

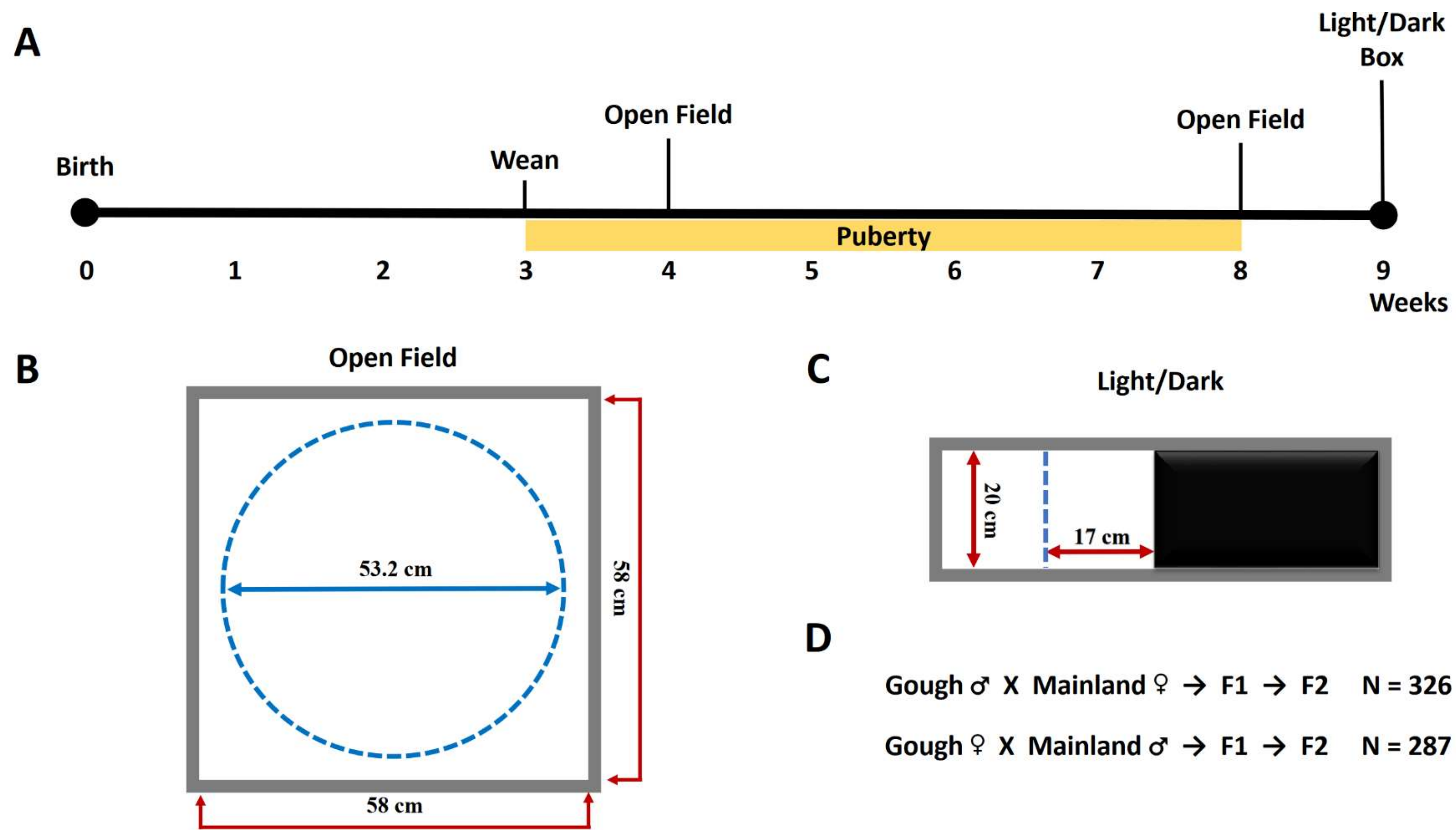

Figure 2.2. Summary of behavioral distributions. Distribution of F2 phenotypes across three assays. Means (+/- 1 standard deviation) of GI mice and means (+/- 1 standard deviation) of mainland mice (extracted from data in Stratton *et al.* (2021)) are noted by green and blue bars, respectively.

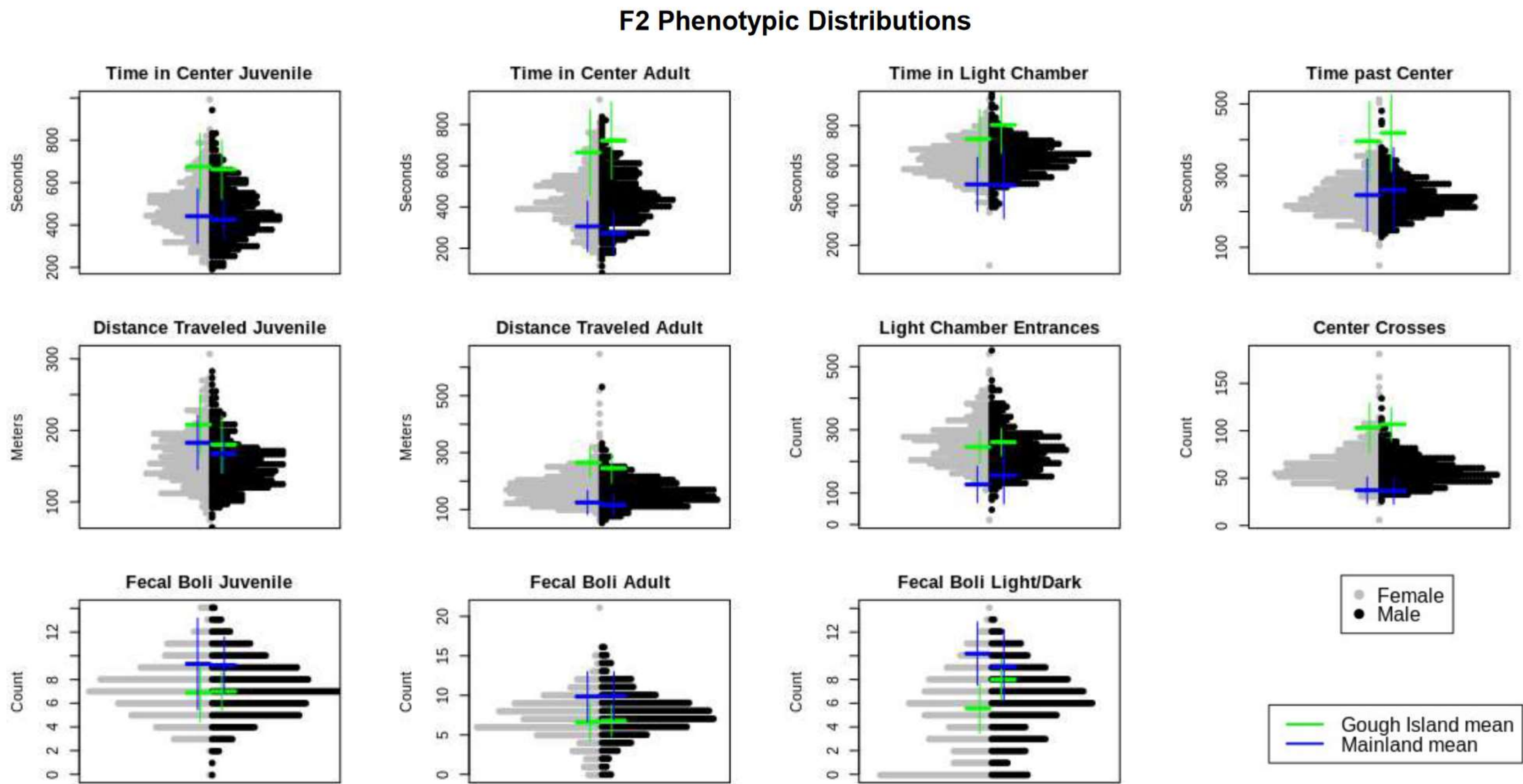

Figure 2.3. Pairwise correlations among body size and behavioral traits across F2s. Correlation significance is denoted by the number of asterisks: * = p-value<0.05, ** = p-value<0.01, *** = p-value<0.001.

Figure 2.4. LOD profiles of QTL identified by single trait mapping. Only chromosomes where a QTL was identified are shown. The maximum 5% significance threshold among traits (4.19) is shown by a dashed line. Thresholds for other traits are similar with a minimum of 3.93.

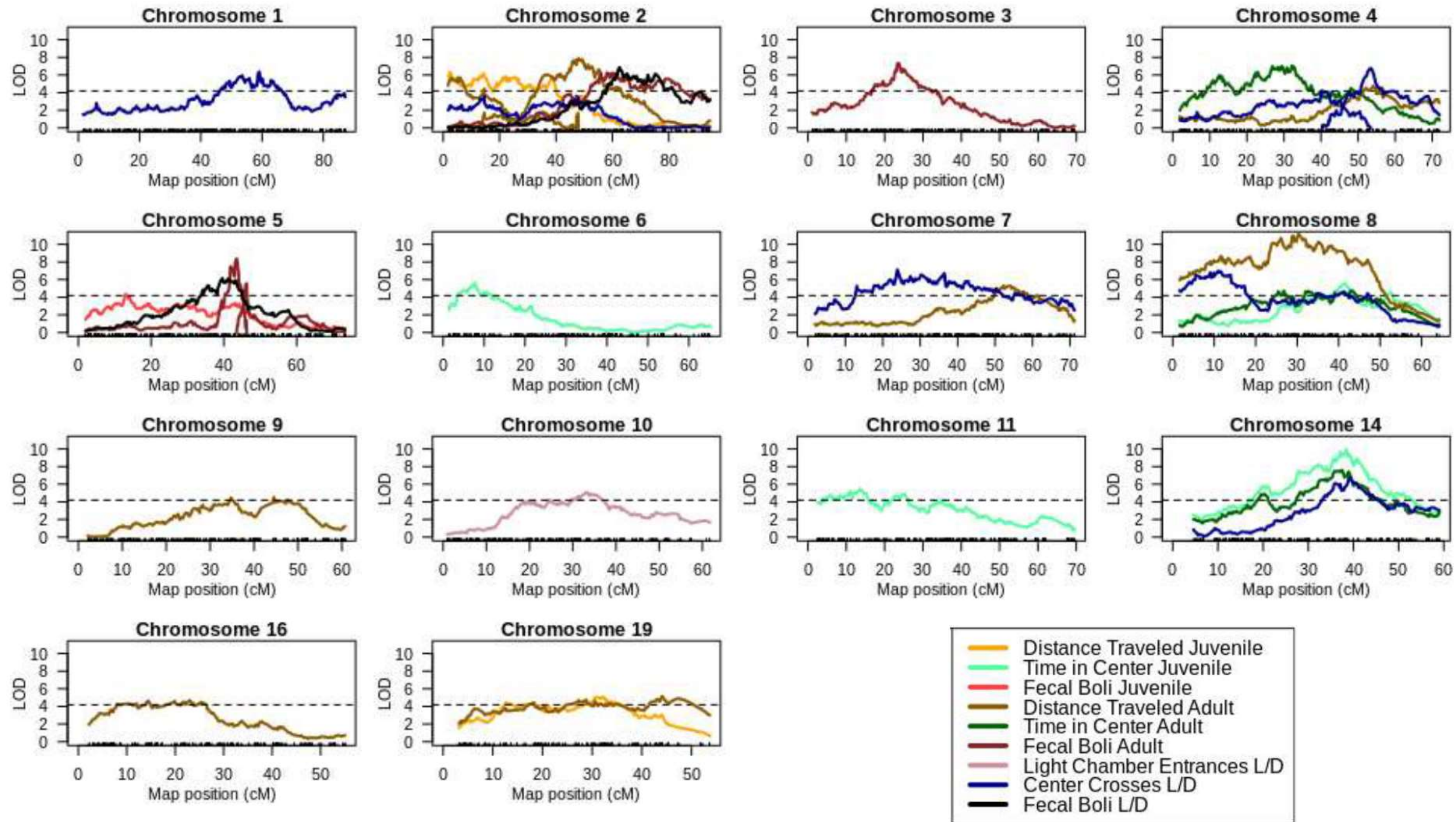

Figure 2.5. Standardized additive effects of QTL. Additive effects of QTL were jointly estimated at every minute of the assay (1 to 30). Each effect was standardized by the phenotypic standard deviation. A. Time spent in the center of the open field as juveniles. B. Distance traveled in the open field as juveniles. C. Time spent in the center of the open field as adults. D. Distance traveled in the open field as adults. E. Time spent in the light chamber of the light/dark box. F. Number of entrances to the light chamber. G. Time spent past the center of the light/dark box. H. Number of crosses past the center of the light/dark box.

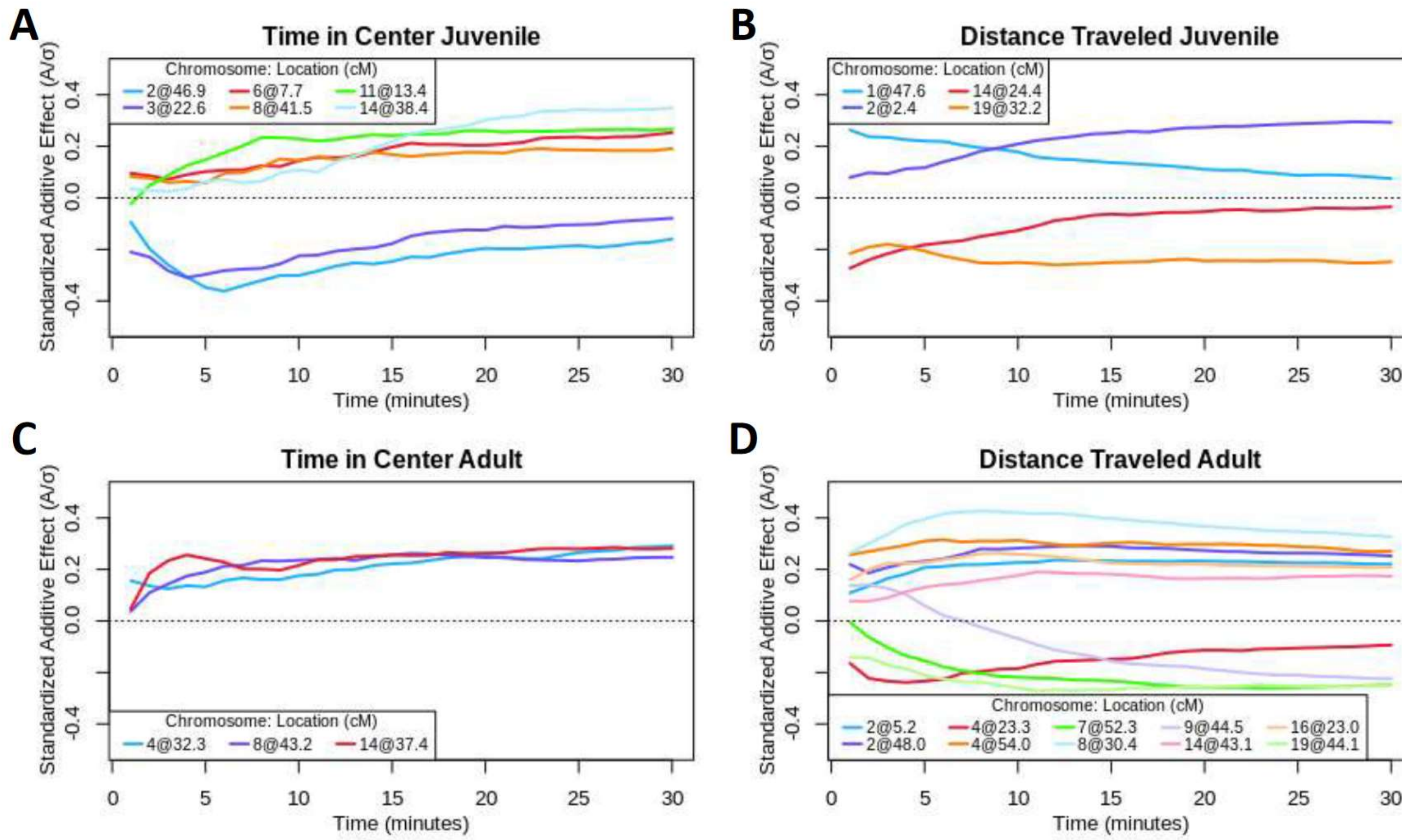

Figure 2.5 continued. Standardized additive effects of QTL. Additive effects of QTL were jointly estimated at every minute of the assay (1 to 30). Each effect was standardized by the phenotypic standard deviation. A. Time spent in the center of the open field as juveniles. B. Distance traveled in the open field as juveniles. C. Time spent in the center of the open field as adults. D. Distance traveled in the open field as adults. E. Time spent in the light chamber of the light/dark box. F. Number of entrances to the light chamber. G. Time spent past the center of the light/dark box. H. Number of crosses past the center of the light/dark box.

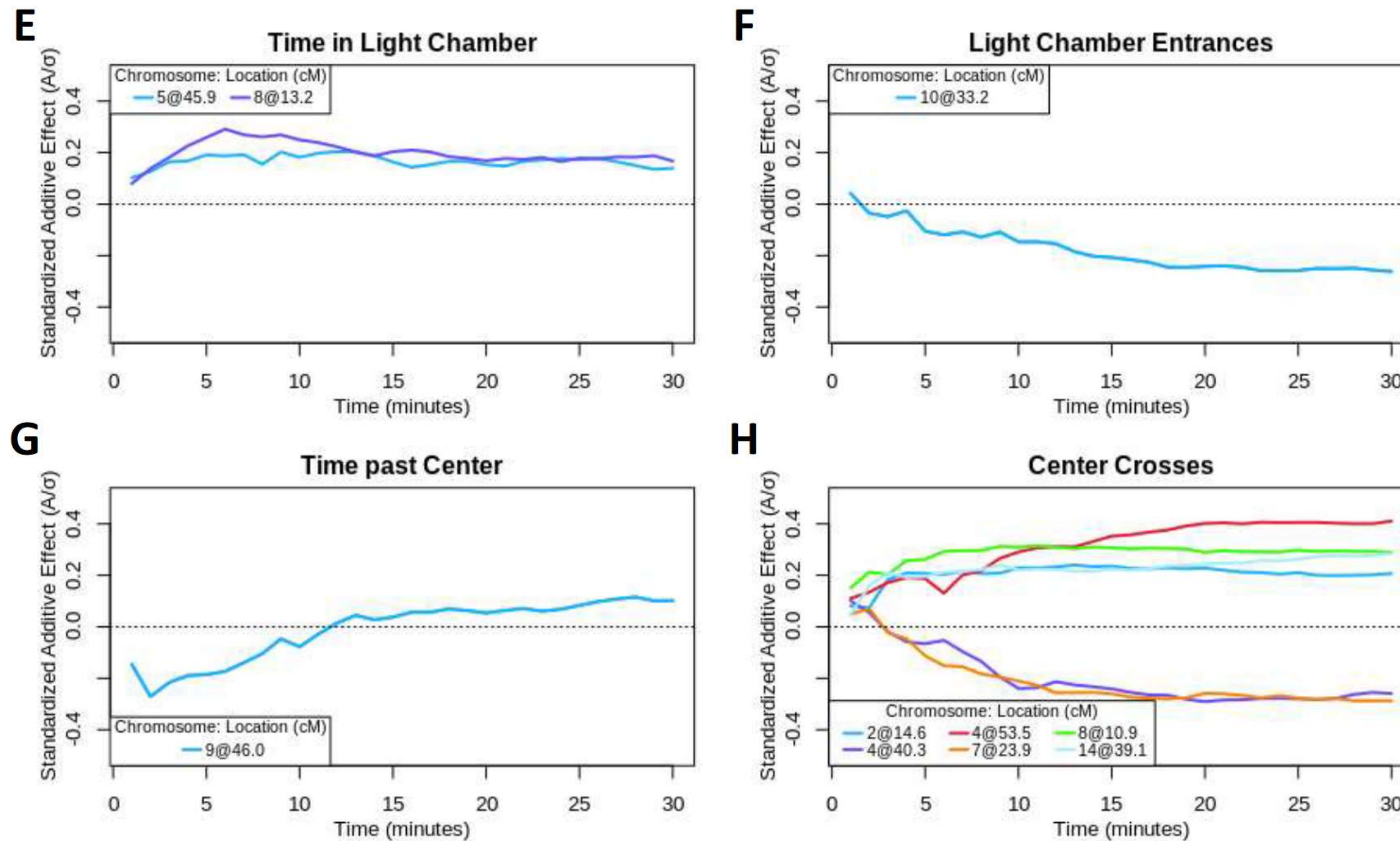

Figure 2.6. Effect plot of female-specific QTL on chromosome 1. The number of center crosses in the light/dark box for males (A) and females (B) within each genotypic class is plotted for the marker at the peak of the QTL. Bars denote means of genotypic classes +/- one standard error. “MM” = homozygous mainland alleles, “GM” = heterozygous, “GG” = homozygous Gough Island alleles.

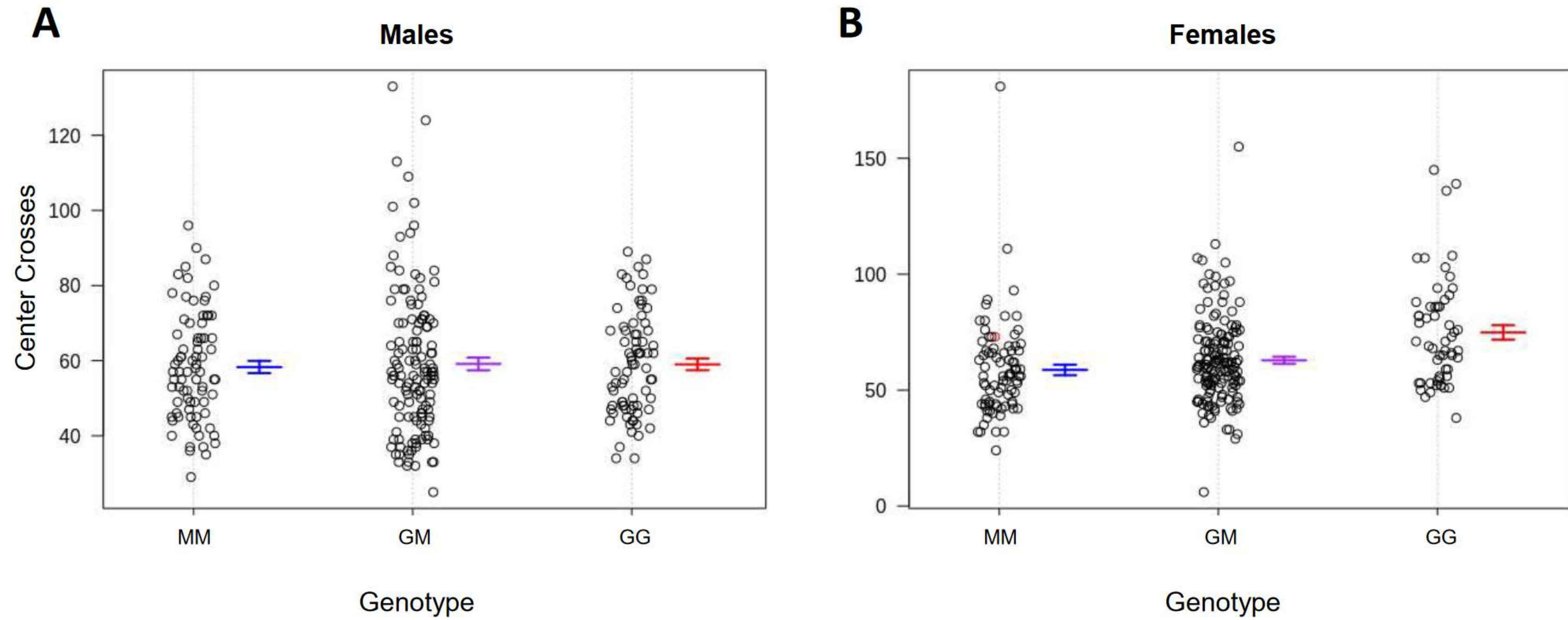

Table 2.1. Summary of QTL for behavior. a. Position of QTL in megabases based off the GRCm39 build of the house mouse genome. b. Position of lower bound in megabases from 1.5 LOD confidence interval. c. Position of upper bound in megabases from 1.5 LOD confidence interval. d. Methods that identified the QTL: "ST" = Single trait analysis, "HK" = multivariate function-valued trait analysis, "SL" = average LOD score of independent QTL scans from function-valued trait analysis, "ML" = maximum LOD score of independent QTL scans from function-valued trait analysis. e. Sign of GI mouse strain mean minus mainland mouse strain mean. Colon indicates change from early to late timepoints of the assay. f. Sign of additive effect of substituting a GI mouse allele. Colon indicates change from early to late timepoints of the assay. The 95% confidence interval of the effect must not overlap zero for a change in sign to be reported. g. Sign of dominance effect (mean of heterozygotes minus the midpoint between the means of the two homozygote classes). If the 95% confidence interval for the effect overlapped zero a "0" is reported.

Assay	Age	Trait	Chromosome	Position (Mb)[a]	Lower Bound[b]	Upper Bound[c]	Methods Detected[d]	Gough Parental Direction[e]	Additive Direction[f]	Dominance Direction[g]
Open Field	Juvenile	Time in Center	2	94.11	88.63	99.32	HK, SL	− : +	−	−
			3	72.58	67.68	81.26	HK	− : +	−	−
			6	27.52	16.05	39.46	ST, ML	− : +	+	−
			8	104.58	91.58	119.60	ST	− : +	+	+
			11	34.38	7.12	67.77	ST	− : +	+	0
			14	90.32	80.55	98.37	ST, HK, ML, SL	− : +	+	+
		Distance Traveled	1	126.2	113.44	137.15	HK	+	+	+
			2	5.99	3.18	69.75	ST, HK, ML, SL	+	+	0
			14	62.13	14.61	79.21	HK	+	−	0
			19	43.32	20.36	49.72	ST, ML, SL	+	−	0
		Fecal Boli	5	30.63	21.77	108.62	ST	−	−	−

	Adult	Time in Center	4	84.68	35.93	91.39	ST, ML, SL	+	+	−
			8	109.4	45.29	118.22	ST, HK	+	+	0
			14	86.92	76.4	93.42	ST, HK, ML, SL	+	+	+
		Distance Traveled	2	11.94	3.18	27.73	ST	+	+	0
			2	99.01	77.16	123.60	ST, HK, ML, SL	+	+	0
			4	57.65	37.55	65.27	SL	+	−	0
			4	133.5	123.81	139.01	ST, HK, ML, SL	+	+	0
			7	129.45	120.16	137.37	ST, ML, SL	+	−	0
			8	78.96	60.36	100.12	ST, HK, ML, SL	+	+	−
			9	105.28	69.38	115.44	ST, HK, ML, SL	+	+ : −	0
			14	99.33	75.05	119.69	ML	+	+	0
			16	45.59	9.07	57.56	ST, HK, ML, SL	+	+	0
			19	57.99	14.58	60.23	ST, ML, SL	+	−	−
		Fecal Boli	2	130.24	113.07	170.75	ST	−	−	−
			3	74.11	71.97	81.94	ST	−	+	−
			5	106.31	103.17	107.39	ST	−	−	−
			5	113.5	110.66	114.64	ST	−	+	+
Light Dark Box		Time in Light Chamber	5	112.86	110.54	130.70	HK	+	+	+
			8	33.7	26	42.02	HK, SL	+	+	+
		Time past Center	9	107.37	91.3	121.17	HK	+	− : +	−
		Light Chamber Entrances	10	91.48	54.07	99.46	ST, ML	+	−	0

		Center Crosses	1	157.98	126.96	167.82	ST (Females)	+	+	0
			2	27.1	3.18	124.85	ST, HK	+	+	+
			4	102.71	57.76	120.17	ST	+	−	−
			4	132.64	129.6	134.31	ST	+	+	+
			7	63.32	54.2	108.92	ST, HK, ML, SL	+	−	−
			8	31.08	10.79	36.30	ST, HK, ML, SL	+	+	0
			14	93.07	79.92	98.37	ST, HK, SL	+	+	+
		Fecal Boli	2	137.4	133.02	164.48	ST	−	−	0
			5	98.66	80.13	108.86	ST	−	−	−

Table 2.2. Standardized additive effects for fecal boli QTL. a. Additive effect of QTL divided by the standard deviation. Sign of effect reflects the action of a GI mouse allele. Note that the GI strain deposited fewer fecal boli than the mainland strain in all assays (see Figure 2).

Assay	Chromosome	Location (cM)	Standardized Additive Effect (A/σ)[a]
Open Field Juvenile	5	13.06	-0.221
Open Field Adult	2	58.161	-0.244
	3	23.363	0.309
	5	43.491	-0.604
	5	46.257	0.379
Light/Dark Box	2	62.55	-0.303
	5	39.586	-0.219

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Chapter 3

Quantitative trait loci governing indirect genetic effects on behavior and body size

Abstract

Phenotypes can be altered by genetic variants in social partners. These indirect genetic effects (IGEs) can change evolutionary trajectories, yet the loci underlying IGEs in natural populations are largely undocumented. We took advantage of house mice from Gough Island (GI), which evolved to be bigger, less anxious, and more exploratory than their mainland relatives, to identify quantitative trait loci with IGEs (iQTL). Using 296 pairs of F2 cagemates derived from intercrosses between GI mice and mice from a mainland strain, we discovered four iQTL wherein the genotype of one mouse affects the body size, anxiety, or exploration of its cagemate. We found the iQTL using a standard intercross design and the same data utilized to detect QTL with direct effects, demonstrating an untapped potential in traditional mapping studies. iQTL and direct QTL for the same phenotypes have similar effect sizes, but are located in distinct regions of the genome, highlighting the need for independent analyses. All iQTL appear only in adults, three iQTL are influenced by sex, and one iQTL is only detected during the first five minutes of a behavioral assay, indicating that IGEs are influenced by life history and are dynamic. Dominance contributes to all iQTL, with one iQTL showing pure underdominance. Three of the four iQTL have effects that oppose the direction of parental differences, suggesting that adaptation may have been hindered by IGEs. Our findings provide a rare characterization of iQTL in a natural population that has recently evolved extreme behaviors and body size.

Introduction

Genetic contributions to complex traits extend beyond direct effects within individuals. Conspecific interactions affect phenotypes, and the social aspect of the environment may have a genetic component (Moore *et al.* 1997; McGlothlin *et al.* 2010; Baud *et al.* 2021a). These indirect genetic effects (IGEs) shape the evolutionary trajectory of populations by: 1) altering rates of evolution away from predictions from traditional models of phenotypic evolution (Moore *et al.* 1997; Wolf *et al.* 1998; McGlothlin *et al.* 2010; Santostefano *et al.* 2017; Fisher and McAdam 2019), 2) facilitating continued evolution of populations when direct genetic variance is exhausted (Moore *et al.* 1997; Wolf *et al.* 1998; Fisher and McAdam 2019), and 3) leading to conflict between individual fitness and population mean fitness (Fisher and McAdam 2019; McGlothlin and Fisher 2022). To obtain a complete understanding of evolution in natural populations, all sources of genetic variance must be considered.

Indirect genetic effects reflect changes in the phenotype of a focal individual due to the genotype of a separate individual. The phenomenon is perhaps easiest to envision in the form of mother-offspring interactions, when heritable maternal traits alter progeny phenotypes (Cheverud 1984; Warrington *et al.* 2019; Wu *et al.* 2021), and in the development of interactive behaviors such as social dominance and aggression (Moore *et al.* 2002; Wilson *et al.* 2009; Santostefano *et al.* 2017). IGEs modify behavior, physiology, and fitness (Cheverud and Moore 1994; Baud *et al.* 2017; Baud *et al.* 2021b), shifts that may cascade to new groups with migration (Makowicz *et al.* 2022). Partner traits that affect focal individuals may include non-social modifications to the environment as well as social influences (Wolf et al. 2011). Accounts of evolutionary histories are incomplete when IGEs are ignored, especially when opportunities for conspecific interactions are pervasive (Fisher and McAdam 2019).

IGEs have been documented in animals and plants – in laboratory conditions, agricultural

settings, and natural populations (Alemu *et al.* 2014; Bailey and Hoskins 2014; Camerlink *et al.* 2015; Baud *et al.* 2017). IGEs may contribute to phenotypic variance in any experiment when individuals are reared in proximity; yet IGEs are typically ignored. Most previous studies characterized the composite effects of IGEs using polygenic models. In several cases, genome-wide association tests have been employed to assign IGEs to locations in the genome (Mutic and Wolf 2007; Ashbrook *et al.* 2015; Ashbrook *et al.* 2017; McGinnis *et al.* 2017; Brinker *et al.* 2018; Wu *et al.* 2019; Hong *et al.* 2020; Baud *et al.* 2021b). Among these examples are loci in *Arabidopsis thaliana* that alter the size, development, and fitness of neighboring plants (Mutic and Wolf 2007), loci in offspring that affect maternal behavior in mouse mothers (Ashbrook *et al.* 2015), loci with IGEs on growth rate in pigs (Hong *et al.* 2020), and a gene in offspring associated with preeclampsia risk in human mothers (McGinnis *et al.* 2017).

Although existing studies report genomic regions with IGEs on a variety of phenotypes, they have limitations for addressing IGEs in an evolutionary context. Some investigations search for IGEs only among loci with direct genetic effects, rather than conducting independent scans (Mutic and Wolf 2007). Genome wide association studies describe associations, rather than testing for co-inheritance of genetic markers with phenotypes (linkage). Most importantly, efforts have focused on laboratory organisms, agricultural systems, and humans. Despite growing awareness that the evolutionary consequences of IGEs can be profound, the identification of IGE loci in natural populations remains rare.

An unusual opportunity to identify IGE loci that influence the evolution of behavior and morphology comes from Gough Island house mice. House mice on Gough Island (hereafter "GI"), a remote island in the middle of the South Atlantic, are bolder and more exploratory than mainland conspecifics when raised in a common environment (Stratton *et al.* 2021). GI mice are also the largest wild house mice on record (Rowe-Rowe and Crafford 1992; Gray *et al.* 2015). These evolutionary shifts adhere to longstanding trends for the evolution of behavior and body size in insular populations (Foster 1964; Blumstein and Daniel 2005; Adler and Levins 1994; Benitez-Lopez *et al.* 2021; Gavriildi *et al.* 2022).

Changes in behavior and size of GI mice were likely driven by ecological factors, including the lack of predators and the absence of human commensals (Rowe-Rowe and Crafford 1992). GI mouse populations are dense (Rowe-Rowe and Crafford 1992), neighboring home ranges overlap by an average of 95% (Cuthbert *et al.* 2016). This demographic feature is connected to island-associated behaviors (Adler and Levins 1994) and increases the frequency of conspecific interactions, setting the stage for IGEs.

In this study, we identify quantitative trait loci with IGEs (iQTL) that modulate behavior and body size in crosses between GI mice and mainland mice. By using a dataset originally created to map QTL with direct genetic effects, we demonstrate that additional loci contributing to evolved traits can be discovered by examining social partners. Our findings extend the characterization of loci responsible for IGEs to a natural population that follows a common evolutionary pattern, the island syndrome.

Methods

Mouse Strains and Husbandry

Inbred strains of GI mice and mainland mice were used to generate the F2 mice examined in this study. In 2009, GI mice were live-caught and shipped to the University of Wisconsin School of Veterinary Medicine Charmany Instructional Facility, where a breeding colony was established (Gray *et al.* 2015). Brother-sister mating was conducted for more than 20 generations to establish an inbred line of GI mice. Mainland mice belong to the WSB/EiJ (hereafter "mainland") inbred strain (JAX stock #001145) founded from breeding pairs caught in Maryland. Mainland mice and GI mice were maintained in the same colony for the same amount of time. GI mice and house mice from the eastern coast of North America are likely descended from Western Europe (Gray *et al.* 2014; Agwamba and Nachman 2022), though the geographic locations of source populations are unknown. F1s were generated by crossing mice from the GI strain and the mainland strain in both maternal directions. F1 siblings were crossed to each other to generate F2s. All mice were housed in an identical manner to those used in Stratton *et al.* (2021), summarized here. Mice were housed in micro-isolator cages with corn cob substrate (1/8th inch; The Andersons Lab Bedding), with *ad libitum* access to food (Envigo 2020X Teklad Global Diet) and water. Breeders were provided with a higher fat chow (Envigo 2019 Teklad Global Diet) and a red mouse igloo (Bio Serv). All cages were provided with nesting material and irradiated sunflower seeds (Envigo). Cage changes occurred every 6-8 days or, in the case of a new litter, 10 days after parturition. The colony was kept in a temperature-controlled room (20-23°C) under a 12-hour light/dark cycle.

Mice used for behavioral testing were weaned at 21 days after parturition or at 20 days in the case of double litters. Mice were housed with one littermate of the same sex, a decision initially made to reduce behavioral effects of within-cage hierarchies (Horii *et al.* 2017). All mice were weighed to the

nearest tenth of a gram during cage changeouts and after the last behavioral assay (i.e., weekly from ages 4-9 weeks old +/- 1 day).

Phenotypic data from 638 F2s among 19 different breeding pairs were generated over the course of this project. Phenotyping was conducted from January 2020 to July 2021. Twenty-five mice were removed following quality control screening for genotypes (see "Genotyping"). For various reasons (e.g., conflicts with the experimenter's schedule, mice escaping the colony), some F2s were subjected to only one or two behavioral tests, creating imbalances in sample sizes among behavioral assays. The final dataset of 613 mice was then trimmed to 296 pairs of cagemates (592 mice) with complete data for analyses.

Behavioral Assays

All behavioral assays were conducted in a room separate from the main colony in a similar manner to Stratton *et al.* (2021) restated here with differences noted. Each subject was tested three times with the following regimen (Figure 1A): open field (4 and 8 weeks old +/- 1 day; Figure 1B), and light/dark box (9 weeks old +/- 1 day; Figure 1C). All tests were conducted during the light phase of the light/dark cycle under white, fluorescent light. Females were scored for stage of estrous cycle according to Caligioni (2009) on the same day as testing, beginning with the second open field test. The order of subjects tested within litters each day was randomized. Each test began by bringing the subjects' cage into the room for a 30-minute acclimation period. The experimenter remained in the room out of sight of the subjects throughout the acclimation and testing periods except for transfer to and from the arena. The relevant arena for each test was placed in the center of the room next to a movable cart where a computer and video recording hardware were located. The arena was cleaned with 70% ethanol after each test. For the open field test at least five minutes was allowed for the ethanol to

evaporate before testing a new subject. For the light/dark test the floor panels were removed and shaken to evaporate the ethanol instead of using a five-minute waiting period. All tests were video recorded using Debut Video Capture Software v 5.33 at default settings with Focus set to 0. A Logitech HD Pro Webcam C920 was positioned directly above the center of the arena. Two videos were taken before each test to assist in video analysis: an "empty" video containing a short recording of the empty arena, and a calibration video containing a short recording of the arena with specific positions marked. The "empty" video was used to align all images of the test recording. The calibration video was used to define coordinates of regions of interest and to convert pixels to millimeters. Subsequent subsections provide additional details of experimental design for each test.

Open Field Test

The open field (58cm W x 58cm D x 58cm H; Figure 1B) was constructed from expanded, white PVC (Grainger Industrial Supply). Lighting in the room was set so that the center of the open field measured 300 +/- 5 lux. A calibration video was taken using a poster board on the bottom of the arena with the center and each corner (1 inch from both edges) marked by circles drawn with black marker. Subjects were initially placed in the center of the arena facing away from the movable cart. The video recording software was started, and the subject was allowed to freely explore the arena. After 30 minutes of uninterrupted exploration, the subject was returned to its home cage and the number of fecal boli in the arena was counted, a commonly used readout of anxiety originally validated in the rat (Hall 1934).

Light/Dark Test

A mouse-sized place-preference chamber (68.6cm W x 20.3cm D x 38.1cm H; San Diego Instruments; Figure 1C) was used for a light/dark test. One half of the arena had a black floor and was

exteriorly covered with black static cling film. The opposite chamber had a white floor and was exteriorly covered (interiorly covered in Stratton *et al.* (2021)) with white static cling film except for the lid to allow video observation. The chamber divider was positioned 4 cm above the floor to allow free access between both chambers and was covered in black static cling film on the side facing the light chamber. Lighting was set so that the center of the light chamber measured 300 +/- 5 lux. A calibration video was taken using a separate floor panel placed on top of the floor in the white chamber with the center and each corner (1.27 cm from both edges) marked by circles drawn with black marker. Subjects were initially placed in the center of the light chamber facing the entrance to the dark chamber. The video recording software was started and the subject was allowed to freely explore the arena. After 30 minutes of uninterrupted exploration, the subject was anesthetized (see "Corticosterone Quantification") and the number of fecal boli in each chamber was counted.

Analysis of Videos

To minimize observer bias, the processing of behavioral videos was automated and blind with respect to mouse ancestry. Videos were translated into x and y coordinates of the subject for each frame of the video using the following pipeline. First, the raw videos were converted using ffmpeg via the following command:

```
ffmpeg -i inputFileName.avi -pix_fmt nv12 -f avi -vcodec rawvideo convertedFileName.avi
```

The converted video file was run through an ImageJ script associated with Mousemove (Samson *et al.* 2015), which we modified to label frames deleted due to inability to maintain the framerate or frames when no mouse was detected. Light/dark videos were manually cropped to view only the light chamber

to speed processing and improve background subtraction. Deleted frames and frames when multiple objects were detected were reincorporated using the position of flanking frames. Calibration videos were run through the same process to obtain positions of known locations in the arena. The tracking software tends to erroneously detect multiple objects. The trajectory files in such instances were manually edited to remove objects that never moved (i.e., non-mouse objects).

Combined trajectory files were then analyzed to measure a variety of different traits depending on the test (see below). Pixels were translated to mm using known distances between objects in the calibration trajectory file. A movement threshold was set for each subject using the method of Shoji (2016). Output for each trait was recorded for every minute of the test for use in function-valued trait analysis.

The center of the open field was defined as a circle with a 26.59 cm radius (14.5 cm radius in Stratton *et al.* (2021)) which stretches to 1 inch from the edge of the arena. Distance traveled was the sum of all positional changes above the movement threshold in the combined trajectory file.

Time spent past two different thresholds in the light chamber were recorded: 1) 1.27 cm (0.5-inch) from the dark chamber, and 2) midpoint of the light chamber. The number of crosses past these thresholds was also recorded.

Corticosterone Quantification

Immediately following the light/dark box test, each mouse was transferred to a glass jar and anesthetized using an isoflurane-soaked gauze. Once unresponsive, the mouse was decapitated, and blood was collected and stored on ice until all tests for the day were completed. Blood was centrifuged at 1200 rpm for 10 minutes to collect plasma. Samples were stored at -20°C until assay submission. Plasma corticosterone concentration for 50 randomly chosen F2s (balanced by sex) was quantified by

ELISA at the Wisconsin National Primate Research Center. Samples exceeding the maximum detection limit of the assay were recorded as “1000 ng/mL”.

Genotyping

F2 carcasses were stored at -20°C following euthanasia. At a later date, carcasses were thawed and liver tissue was collected and stored at -20°C. Later, all liver samples were submitted to Neogen for genotyping on the Giga Mouse Universal Genotyping Array (https://www.neogen.com/categories/genotyping-arrays/gigamuga/). The array contains over 143,000 markers selected to provide maximal information for studies utilizing the Collaborative Cross (Aylor *et al.* 2011; Threadgill and Churchill 2012), and Diversity Outbred Cross (Svenson *et al.* 2012) with additional markers selected to capture diversity in wild mouse populations. Previous versions of this array were used successfully to map QTL for morphological traits between GI mice and WSB/EiJ mice (Gray *et al.* 2015; Parmenter *et al.* 2016; Parmenter *et al.* 2022).

Quality Control Checks

Several controls were used to identify and remove samples and markers showing patterns that suggested errors. All parents (F1s) and grandparents (GI and mainland) of the F2s were included in the genotyping to assess residual heterozygosity within inbred strains and assist in quality control checks. The grandparents of the F2s were submitted in duplicate to assess the genotyping error rate. Rates of missingness and signal intensity among plates were analyzed to assess plate performance.

Genotype calls on the sex chromosomes and mitochondria were checked to ensure that each sample’s genotype met expectations based on the recorded sex and pedigree (i.e., cross direction) of

the mouse. Four mice with mismatched phenotypic and genotypic sex predictions were removed. An additional 20 samples were removed based on the following criteria: 1) over 10% missing data among informative markers, or 2) over 2% missing data among informative markers and abnormally high signal intensity variance among all markers.

Marker-level checks were performed on the remaining dataset of 614 mice. Markers with missing data in over 30 samples or heterozygous calls in under 270 samples were removed. Evidence for segregation distortion was evaluated using the geno.table() function in R/qtl. Following the method outlined in Broman and Sen (2009), markers with a p-value less than 1e-7 were removed. A genetic map was built using the Carter-Falconer map function assuming a 0.1% genotyping error rate. The first marker on chromosome 18 was subsequently removed as it appeared unlinked to the rest of the chromosome. Genotyping error LOD scores were calculated using the calc.errorlod() function. Calls with error LOD scores of 5 or higher were replaced with NAs. The number of inferred crossovers for each mouse was calculated. One additional sample was removed at this step due to a high number of inferred crossovers.

The remaining dataset was trimmed to F2s with complete data for both cagemates. The final dataset included 296 pairs of F2 cagemates with genotype information at 31,681 informative markers across all autosomes and the X chromosome.

Estimation of Correlations between Cagemates

To mitigate the impact of similar maternal environments experienced by social partners, each phenotype was first fitted to a linear model with litter ID as a categorical predictor. Residuals from fits of this model were used in the estimation of Pearson's correlations between cagemates for all pairs of

traits. As only 50 samples were assayed for corticosterone concentration, mother ID was used in place of litter ID as a categorical predictor. For correlations involving the same trait in both cagemates, the mouse with the lower ID number (assigned at one week of age before cagemates were determined) was arbitrarily designated as cagemate "A" to prevent an artificial mirroring of data by using each value in both the x and y dimensions.

iQTL Analyses

All statistical analyses were conducted in R (v 4.1.3) (R Development Core Team 2022). To search for iQTL, phenotypes of mice were compared to the genotypes of their cagemates (Figure 1D). Phenotypes considered in iQTL analyses included weekly body weights from four to nine weeks of age, juvenile and adult open field traits (time spent in the center, distance traveled, and number of fecal boli deposited), and light/dark box traits (time spent in the light chamber, number of light chamber entrances, time spent past the center of the light chamber, number of crosses past the center of the light chamber).

We employed two strategies to identify iQTL for behavioral traits: 1) single trait analyses using the value of each phenotype at the end of the behavioral assay, and 2) function-valued trait analyses combining information across the entire behavioral assay. iQTL scans for body weights and fecal boli counts were restricted to single trait analyses. Recognizing that iQTL could be sex-specific, we conducted all analyses in triplicate: 1) using the entire dataset, 2) using only females, and 3) using only males. For single trait analyses, we also conducted scans with sex treated as an interactive covariate. Interactive covariates are not implemented for the package we used for function-valued trait analyses.

Single Trait Mapping

Single trait analyses (hereafter "ST") were conducted using the value of each behavioral trait from the culmination of the assay. To begin, genotype probabilities for each mouse were calculated using the calc.genoprob() function in R/qtl assuming a 0.1% genotyping error rate. Genotype probabilities were calculated at every marker and at a set of additional pseudomarkers to ensure probabilities were present at every 0.2cM. Single-iQTL analysis was conducted using Haley – Knott regression (Haley and Knott 1992). Thresholds for iQTL significance were calculated using a permutation test (Churchill and Doerge 1994). iQTL with LOD scores in the top 5% among 1,000 permutations were deemed significant. To determine which covariates to include in the iQTL scan, environmental and pedigree-related (i.e., mother identity) predictors associated with the behavior of the focal mouse were evaluated using linear models for each trait. An independent variable was included as an additive covariate when deemed significant by an additional sum-of-squares test comparing models that included or excluded the variable. Covariates used for each trait are listed in Supplementary Table 3. Sex was included as an additive covariate for all traits in scans where it was treated as an interactive covariate. To increase power to detect iQTL, we also included as additive covariates genotypes at estimated peak positions of direct QTL for the same behavioral and body weight phenotypes mapped in the same intercross. Including genotypes at direct QTL as covariates accounts for the direct effects of a locus while allowing IGEs of the locus to be independently tested. Direct QTL were identified using the same approach as iQTL, except each mouse's phenotype was compared to its own genotypes, across a total of 613 F2s (Figure 1D). The direct QTL for behavior are reported in Stratton *et al.* (2023). Direct QTL for body size are reported in Supplementary Table 4.

Function-valued Trait Mapping

To test for iQTL effects at restricted timepoints of each behavioral assay, we conducted function-valued trait QTL mapping as implemented in r.funqtl (Kwak *et al.* 2015). Fecal boli were counted only at the end of each assay and were not included in this analysis. Values for each behavior were calculated at each minute of the test (1-30) and used as input phenotypes. Phenotypes were smoothed using B-splines. The collection of functional principal components (fPCs) that explained over 99% of the variance in the data were extracted. The fPCs were then fit to the same covariates used in ST analyses and residuals were extracted for iQTL mapping. Since ST analyses produced no evidence of X-linked iQTL, the X chromosome was excluded from function-valued trait mapping to improve computational speed and compliance with the software. Three different mapping approaches were used: 1) "HK" multivariate mapping following Knott and Haley (2000), 2) "SL" mapping, which takes the average LOD score across scans of each fPC individually, and 3) "ML" mapping which takes the maximum LOD score across scans of each fPC individually. These three approaches are designed to capture QTL with a wide range of effect sizes and durations. Permutations to establish significance thresholds were conducted in a similar manner to the ST analyses using the scanoneM() function with the appropriate mapping method specified.

Cagemate Permutation Analysis

To assess the likelihood of identifying spurious iQTL across all scans, we conducted a cagemate permutation analysis. Briefly, each permutation replicate randomly assigns cage identity to each mouse within the sexes. All scans for iQTL are then repeated using these permuted cagemate assignments and false positive iQTL are identified using the same thresholds for significance determined from the original analysis. A *P* value is then calculated by determining the number of permutations that yielded the same number or higher of false positive iQTL that we found in the original analysis.

Estimation of QTL Effects

Additive and dominance effects of QTL were estimated using the fitqtl() function in r.qtl. Covariates for each trait (including direct QTL) were included during effect estimation. Sex and sex-by-iQTL interaction effects were included for the iQTL on chromosome 13 for cagemate time in the light chamber. Females are the default sex. To improve comparisons among iQTL, the additive effect of each locus was standardized by the phenotypic standard deviation. The standard error of the dominance to additive ratio (D/A) was estimated using the delta method (Lynch and Walsh 1998).

Candidate Gene Nomination

Candidate genes for each iQTL were nominated based on two primary criteria: 1) association with abnormal social behaviors in mice, and 2) sequence differences between the two inbred strains used in the cross. To begin, the 1.5 LOD confidence interval for each iQTL was used to extract a list of genes from the Mouse Genome Informatics (MGI) database (http://www.informatics.jax.org/marker/). We searched for "protein-coding genes" using the code "MP:0002557" corresponding to "abnormal social/conspecific interaction behavior" under "Phenotypes/Diseases" to gather an initial list. All genes from these initial lists are found in Supplementary Table 1. For these genes, we extracted sequence differences between the two inbred strains used as parents in the cross using the UCSC Variant Annotation Integrator Tool (http://genome.ucsc.edu/cgi-bin/hgVai) applied to genome sequences of GI mice (Nolte *et al.* 2020) and WSB/EiJ mice (Keane *et al.* 2011). We used two different searches to collect sequence differences of interest. In the first search, we filtered for "CDS – nonsynonymous", "exon loss caused by deletion", and "splice site or splice region" within "Functional roles". In the second search, we included all functional roles and filtered for elements conserved across placental mammals. Intergenic variants were associated with a gene when they resided within 100kb of the start or end sites

of the gene.

Data Availability

Raw measurements and associated metadata for each F2 used in iQTL analyses are included in Supplementary Table 5.

Results

Cagemate Phenotypes Influence Behavior and Body Size

Phenotypic correlations among cagemates highlight the potential for IGEs. Correlations adjusted for litter effects are context-dependent, varying by trait, sex, and age (Figure 2). All correlations are sex specific. Time spent in the center of the open field is negatively correlated in juvenile females (Pearson's r = -0.27; P < 0.001), suggesting that cagemates often have different levels of anxiety. Body weight is negatively correlated with time spent past the center of the light chamber in male cagemates (r = -0.16; P < 0.01), suggesting that larger males impose greater anxiety on cagemates. Body weights of female cagemates are positively correlated at all ages. In females, 8 of 17 phenotypes show within-trait correlations between cagemates (Figure 2A, along the diagonal), whereas no phenotypes display within-trait correlations among male cagemates (Figure 2B, along the diagonal). All but one of the correlations among males are negative, while females show both positive and negative correlations in similar proportions. Males do not show correlations involving traits measured before adulthood.

In females, corticosterone concentration is negatively correlated with the time a cagemate spends in the light chamber (Figure 3A; r = -0.47; P < 0.05) and negatively correlated with the time a cagemate spends past the center of the light chamber (Figure 3B; r = -0.42; P < 0.05). These correlations are similar after adjusting for mother effects (r = -0.44 and r = -0.36 for time in light chamber and time past center respectively). In contrast, corticosterone concentration is not correlated with any cagemate traits in males.

Together, our results show that cagemates affect each other's behavior and body weight. Sex and age play important roles in this phenomenon.

iQTL Exert Indirect Genetic Effects on Cagemate Phenotypes

We identified four iQTL that influence cagemate body weight or behavior (Table 1; Figure 4). All iQTL are unique to adults. An iQTL on chromosome 4 affects cagemate body weight at 9 weeks of age in both sexes. An iQTL on chromosome 13 regulates time spent in the light chamber and interacts with sex. An iQTL on chromosome 11 modifies the time male cagemates spend in the light chamber. Function-valued trait mapping (using the "ML" criterion) uncovered an iQTL on chromosome 14 that changes the distance female cagemates travel. None of the iQTL overlap in position with direct QTL modulating the same phenotypes in the same cross (Figure 5).

Although the number of detected iQTL is small, iQTL show signs of genetic complexity (Figure 6; Table 1). All iQTL display evidence of dominance. The iQTL on chromosome 11 is underdominant, with male cagemates of heterozygotes spending less time in the light chamber than male cagemates of both homozygotes (Figure 6B, right panel). This iQTL has no detectable additive effect. Across iQTL, either GI mouse alleles are recessive, or heterozygotes show lower trait values.

Additive effects of iQTL are modest and mixed in their direction. At the iQTL with the largest additive effect, substituting a GI mouse allele reduces the time female cagemates spend in the light chamber by 0.296 phenotypic standard deviations (29.3 seconds), an effect that opposes the phenotypic difference between the parents of the cross (GI mice spend more time in the light chamber, reflecting their increased boldness). The iQTL on chromosome 4 also acts against the parental difference, with the GI mouse allele decreasing cagemate body weight by 0.063 standard deviations (1.7 grams). In contrast, the GI mouse allele at the iQTL on chromosome 14 enhances the distance traveled by female cagemates by 0.154 standard deviations (1.5 meters), aligning with the parental difference. Interestingly, estimated effects of this iQTL are maximized at the beginning of the open field assay (Supplemental Figure 1).

Whereas the three iQTL associated with behavior are influenced by sex and explain between 5.2% and 7.8% of the phenotypic variance, the one iQTL for body weight acts similarly in both sexes and exerts a smaller phenotypic effect, explaining 1.4% of phenotypic variance (Table 1). Importantly, these effect sizes of iQTL are similar in magnitude to effects sizes of direct QTL mapped in the same cross (Figure 7). Among QTL for behavior, iQTL on average explain larger amounts of the phenotypic variance than dQTL (Wilcoxon rank sum test; $P < 0.05$).

Cagemate permutations suggest that the iQTL we identified are likely to be spurious. Among 200 permutation replicates, 156 identified 5 or more false positive iQTL yielding a P value of 0.78 (the iQTL on chromosome 13 was significant in a female specific scan and when sex was treated as an interactive covariate). However, the amount of time spent in cohabitation and change in social dynamics among adults are likely to increase the effects of iQTL suggesting that iQTL in later ages are more likely to be real. Among scans in mice 8 weeks or older, 108 permutations identified 5 or more false positive iQTL ($P = 0.54$).

Genes Associated with Abnormal Social Behavior Colocalize to iQTL Regions

We identified 22 genes associated with “abnormal social behavior” (see “Methods”) underneath iQTL (Supplementary Table 1). Among these genes, we nominate ten (Table 2) as strong candidate genes for iQTL. These ten genes harbor at least one sequence difference between the parental strains used in the cross at sites conserved across placental mammals. *Cmya5,* located within the iQTL for the time female cagemates spend in the light chamber, contains a remarkable 30 amino acid substitutions in the myospryn protein. All amino acid substitutions occur in exon 2 which contains 84% of the protein coding sequence but does not contain any of the annotated functional domains (which reside at the C-terminus). In addition to showing impaired cardiac function, male mice deficient in myospryn display reduced sociability, decreased exploration, lower startle activity, and anhedonia (Tsoupri *et al.* 2021).

While the iQTL on chromosome 14 that affects distance traveled in female cagemates does not overlap genes with known connections to abnormal social behavior, only 23 genes lie within the iQTL interval and harbor sequence changes of interest (Supplementary Table 2).

Discussion

We provide a rare characterization of IGEs connected to a natural population that has evolved unusual behavior and extreme body size. The iQTL we identified have effect sizes comparable to direct QTL for behavior and direct QTL for body weight discovered in the same cross, underscoring the contributions of IGEs to phenotypic evolution on islands. Since housing mice with single social partners (i.e., cagemates) generates detectable iQTL, the number and effects of iQTL in GI mice are possibly larger in the wild. While the forced cohabitation of cagemates in our study may have accentuated IGEs compared to wild house mice, island populations, including GI mice, commonly reach higher densities than mainland populations, thereby increasing the frequency of social interactions (Fisher and McAdam 2019). Our results demonstrate that consideration of multiple sources of heritable variation can refine evolutionary interpretations.

Cagemate permutations suggest that the iQTL we detected are likely to be spurious. This finding highlights the challenge of identifying iQTL in standard intercross populations. While the sample size of our cross is larger than most QTL mapping populations, studies identifying loci responsible for indirect genetic effects typically have thousands of individuals (Baud *et al.* 2021b). Despite our lack of statistical power, there are two reasons to suspect the iQTL we identified are real. First, the iQTL we identified are only in adults. Since the amount of time spent in cohabitation is likely to increase the effects of iQTL, it is more likely for iQTL to appear in later ages. Second, the effect sizes of the iQTL we found are large (Table 1; Figure 7). As our analysis parallels the method applied to the detection of dQTL for the same traits, we believe it is unlikely that false positive iQTL would yield effect sizes higher than those of dQTL. Further evaluation of these iQTL is warranted, including verification through orthologous approaches. We provide a commentary here on the implications of our findings given that they represent true associations between cagemates.

Patterns of phenotypic correlations between social partners inform inferences about the evolutionary trajectories of GI mice. Body weights of female cagemates are positively correlated across a developmental time span of six weeks (Figure 2A); these correlations could have accelerated the response to selection for increased body size beyond expectations involving only direct genetic effects (McGlothlin *et al.* 2010). A possible biological explanation for body weight correlations among female cagemates is the improved ability to thermoregulate when social partners are larger (Heldmaier 1975). This benefit may not accrue in males whose relatively higher aggression could reduce huddling behavior (Tan *et al.* 2019). In this scenario, the increase in body size due to larger social partners is masked when aggression is also high. Such interaction effects between traits in social partners are sometimes described as "intergenomic epistasis" (Linksvayer 2007; Heath 2010; Wade 2021). Most phenotypic correlations between male cagemates are negative, suggesting the multivariate evolution of behaviors and body size in GI mice may have been hindered by IGEs. We also found that corticosterone concentrations in females following the light/dark box test are negatively correlated with the time their cagemates spend in the light chamber. This result suggests that the anxiety of one mouse affects its social partner, a process known as behavioral contagion (Hatfield *et al.* 1993). While other measures of anxiety do not show similar correlations between cagemates, corticosterone levels might capture a specific form of stress caused by experimenter handling immediately preceding blood collection, which could be analogous to aggressive interactions between cagemates. By accounting for similar maternal environments between cagemates, we increased the chance that the correlations we report are driven by genetic factors; however, we cannot rule out environmental causes. In contrast, iQTL represent direct relationships between genetic inheritance and phenotypic shifts, providing clear insights on evolutionary histories.

Effects of the iQTL we discovered highlight the complex ways in which IGEs evolve. At two iQTL, GI mouse alleles reduce the values of traits predicted to be adaptive on the island. Variants at these

iQTL might have inhibited the response to natural selection in GI mice. All iQTL display evidence of dominance, with one iQTL acting in a purely underdominant manner. Compared to inbred lines of chickens, crossbred chickens showed increased rates of mortality from social partners (Peeters *et al.* 2012), suggesting nonadditive contributions to IGEs that affect cagemate aggression. The connection between dominance and the evolutionary impact of IGEs is understudied (Walsh and Lynch 2018). It is theorized that dominance in IGEs can contribute to the response to selection in social groups depending on the degree of relatedness between interacting individuals (Wright 1986; Wolf *et al.* 1999). A better understanding of dispersal habits and population structure of GI mice could reveal new insights into how IGEs shaped their evolutionary history.

Relatedness between social partners is not the only biological factor that can modify the impact of iQTL. The depth of phenotypic characterization in our study reveals roles of life history and dynamic behavior in IGE prevalence. Three of the iQTL we identified are influenced by sex. Sex-specific IGE loci were reported by Baud *et al.* (2021b), who found that variants associated with stress-coping and wound healing in laboratory house mice were only apparent in male cagemates. Furthermore, we detected no iQTL in juveniles, raising the possibility that social behaviors affecting cagemate phenotypes are unique to adults. Finally, the effects of the iQTL on chromosome 14 are maximized in the initial minutes of the open field test, suggesting the impact of social interactions can degrade rapidly following social isolation. Collectively, our findings indicate that social interactions and the contributions of IGEs are dynamic, even in controlled laboratory conditions where territories are fixed and access to food is unlimited.

One advantage of our approach to identifying iQTL is that it makes no assumptions about the phenotype(s) that mediate IGEs. Alternative strategies centered on certain aspects of social behavior could miss the iQTL we detected. The downside of our agnostic strategy is that it leaves unexamined the phenotypic mechanism responsible for IGEs. The iQTL we identified do not colocalize with direct

QTL found in the same mapping population, suggesting the causal traits are not ones we measured. Behaviors that create or alter social interactions seem more likely to drive the iQTL we found. Among the forms of social interaction known to be affected by candidate genes located in iQTL intervals (Table 2), aggression is likely the most intense. Reductions in aggression are commonly associated with island populations (Adler and Levins 1994; Baier and Hoekstra 2019). Based on injury reports from our laboratory colony, we suspect GI mice are less aggressive than mice from the mainland reference strain. Uncovering the phenotypes that mediate the IGEs we report ultimately will require detailed characterization of social interactions within cages.

Though the causes of the iQTL we detected are unknown, our findings nevertheless demonstrate that it is feasible to map IGEs with conventional methods of QTL analysis. The dataset we used was initially designed to identify direct QTL that affect behavior. Mapping iQTL required no additional data. Housing each mouse with exactly one littermate of the same sex enabled direct tests for coinheritance between the genotype of a focal mouse and the phenotype of its cagemate, avoiding complications that arise from variation in the number and type of cagemates (Baud *et al.* 2021b). Therefore, careful experimental design makes the characterization of iQTL straightforward in traditional mapping populations. We recommend that investigators dissecting the genetics of complex traits consider standardizing the social environment and scanning for iQTL. Reconceptualizing IGEs as detectable sources of phenotypic variation rather than environmental noise promises to improve understanding of genotype to phenotype relationships.

Our study provides a novel characterization of QTL arising from social interactions in a natural population. The detection of QTL responsible for IGEs is feasible using traditional methods of genetic mapping and widens perspectives on long-standing evolutionary patterns. We encourage others to map indirect sources of genetic variation in natural populations so that loci responsible for IGEs in the wild may be compared. A holistic understanding of the adaptive process requires consideration of the

complexities of natural environments in which social interactions play a major, and sometimes heritable, role.

Figure 3.1. Summary of behavioral phenotyping timeline and assays. A. Timeline of a subject mouse's life. B. Schematic of the open field arena. The center defined during video analysis is outlined by the dashed circle. C. Schematic of the light/dark box. During the test, the subject had free access to the two equally sized chambers. The center threshold (used to calculate "time past center" and "center crosses") is noted by the dashed line. D. Schematic of QTL mapping approaches. Direct QTL (dQTL, blue) are mapped using genotype and phenotype associations within the same individual. Indirect QTL (iQTL, red) are mapped using associations between the phenotype of one cagemate and the genotype of the other cagemate.

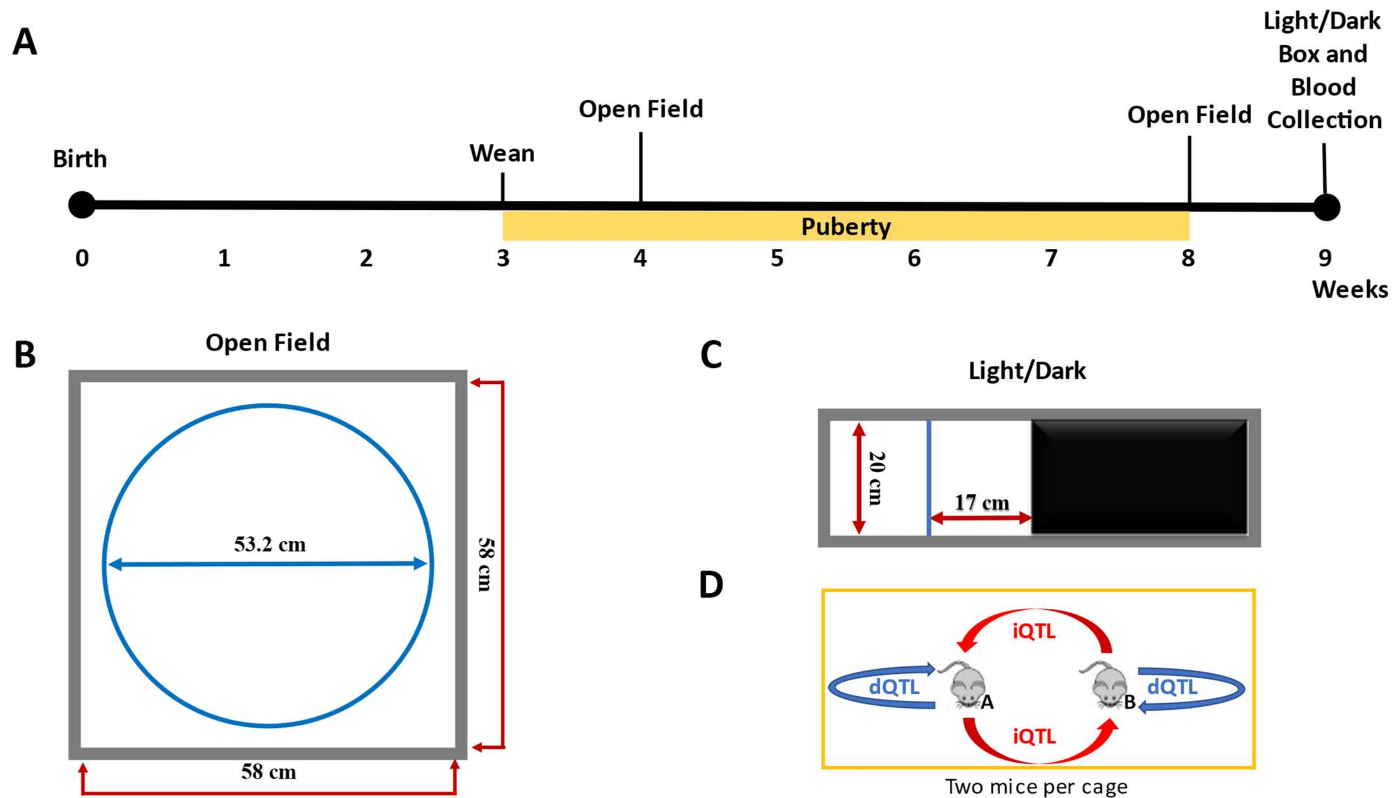


Figure 3.2. Phenotypic correlations between F2 cagemates within sexes. Correlation significance is denoted by the number of asterisks: * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. Correlations involving the same trait (diagonal) were computed using half the sample to avoid duplication of data values. In such cases, the mouse with the lower ID was arbitrarily chosen as cagemate A. All phenotypes were first corrected for litter effects to minimize contributions from a common rearing environment (see "Methods"). Label colors denote behavioral traits measured at the same age as a corresponding weekly body weight. A. Correlations among traits between female cagemates. B. Correlations among traits between male cagemates.

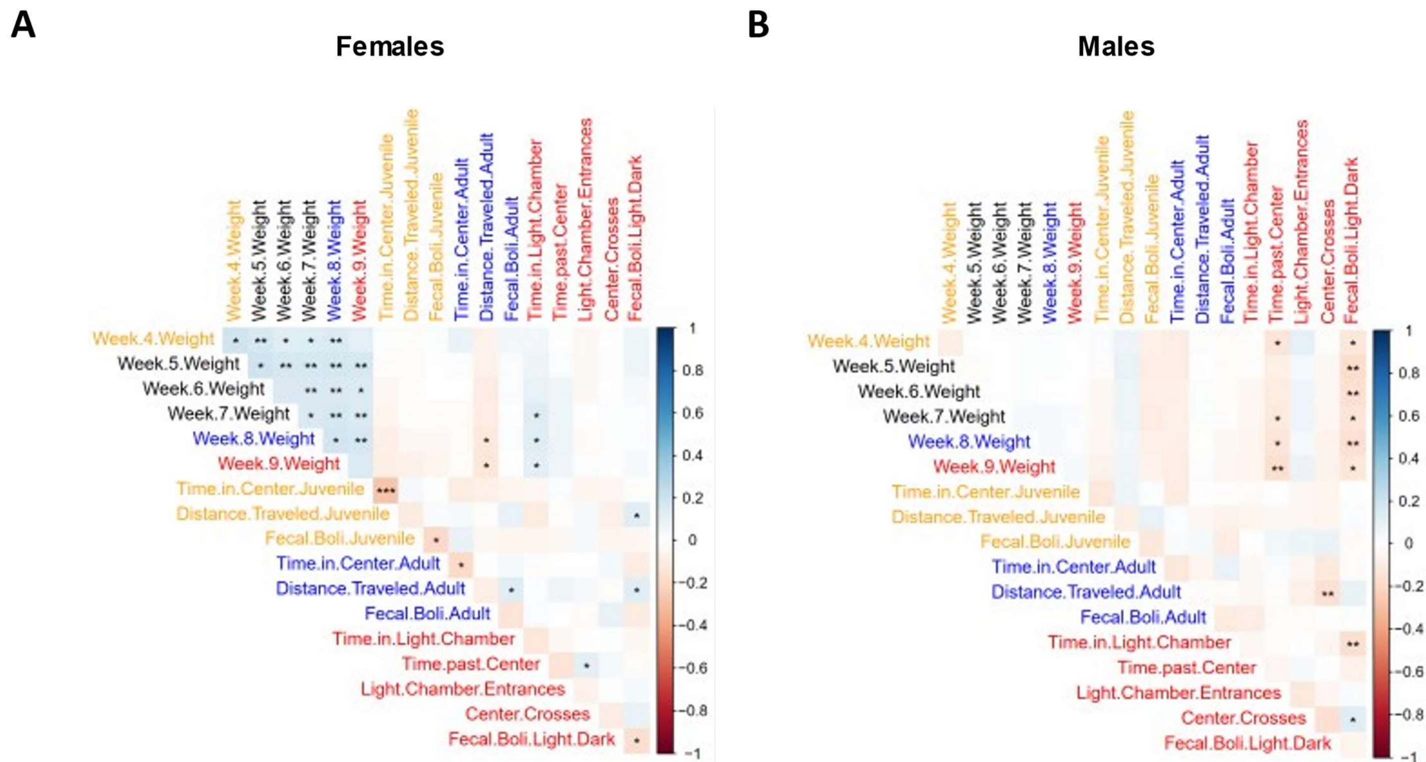
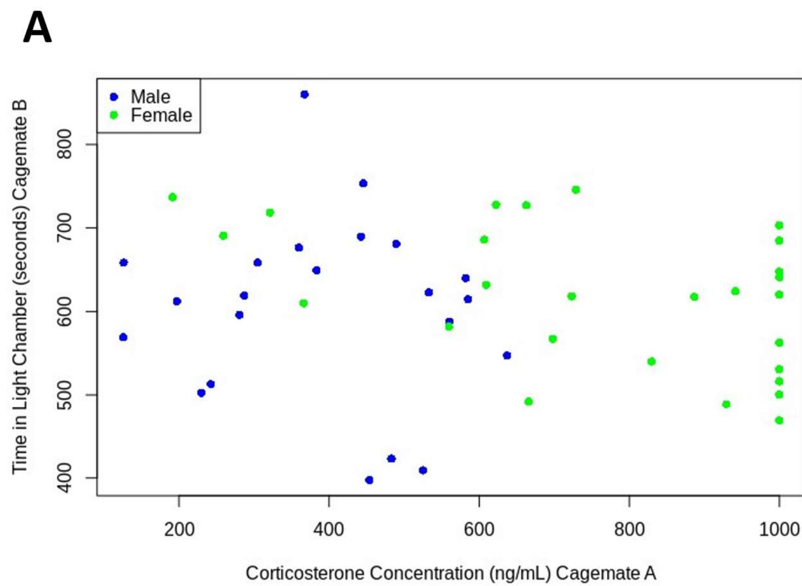


Figure 3.3. Correlations between corticosterone concentration and light/dark box behaviors. Datapoints are colored according to sex. The assay for corticosterone concentration reaches a maximum value at 1000 ng/mL. A. Corticosterone concentration following the light/dark test vs. time the cagemate spent in the light chamber. B. Corticosterone concentration following the light/dark test vs. time the cagemate spent past the center of the light chamber.



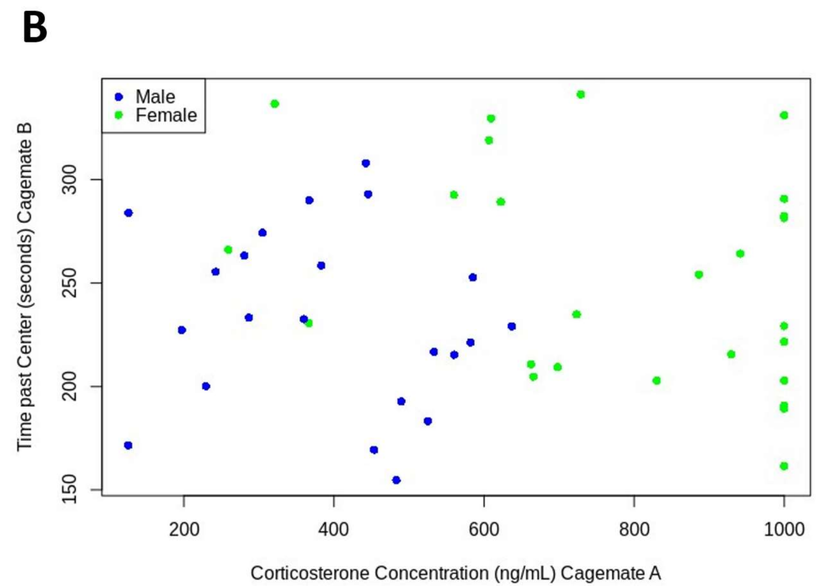
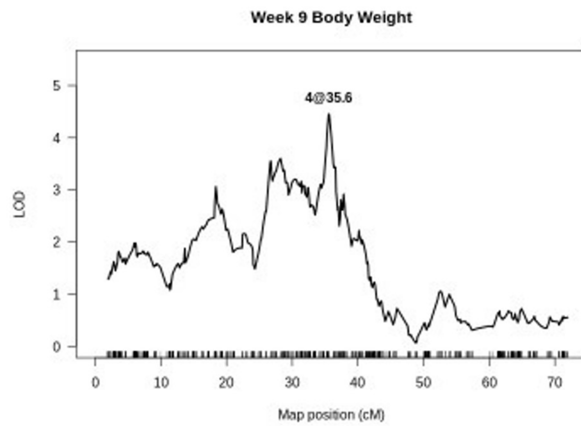
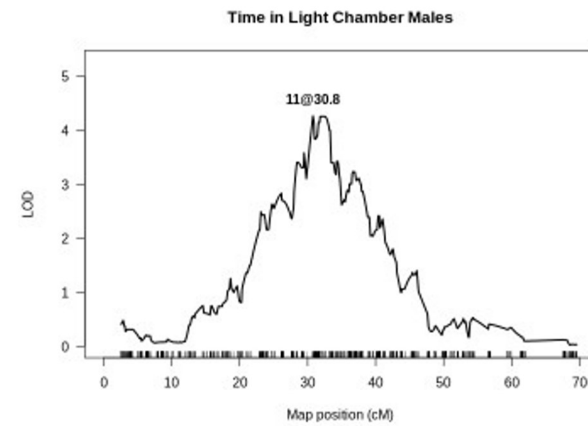


Figure 3.4. LOD profiles of iQTL associated with cagemate phenotypes. The peak of each LOD profile is designated by "chromosome number @ cM position". A. iQTL for cagemate body weight at 9 weeks of age. B. iQTL for time a cagemate spent in the light chamber in adult males. C. iQTL for time a cagemate spent in the light chamber, detected as a sex-by-genotype interaction. D. iQTL for distance a cagemate traveled in the first five minutes of the open field test in adult females.

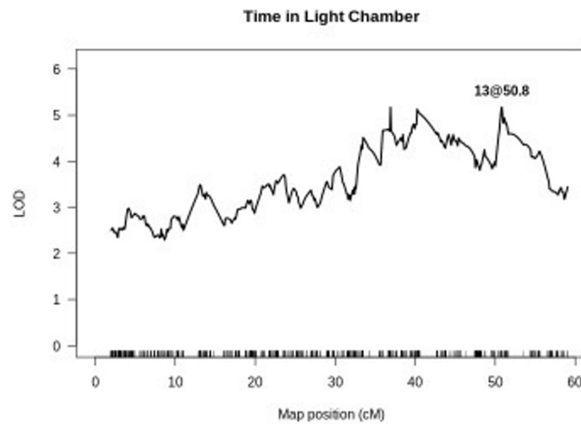
A



B



C



D

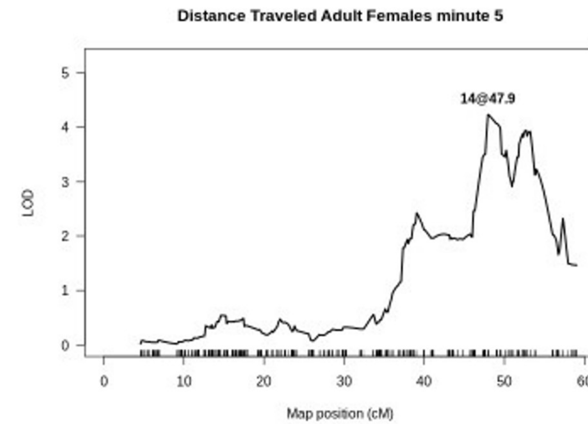


Figure 3.5. Genomic positions of iQTL and direct QTL (dQTL) identified using the same set of phenotypes. Each dot represents the peak position of a QTL. QTL are categorized by whether they affect behavior or body size. QTL detected for the same trait at multiple ages (body size, open field behaviors) were trimmed to the timepoint when the QTL explains the maximum amount of phenotypic variance. Direct QTL for behavior with peak positions located within 5cM are collapsed into a single dot at the average cM position.

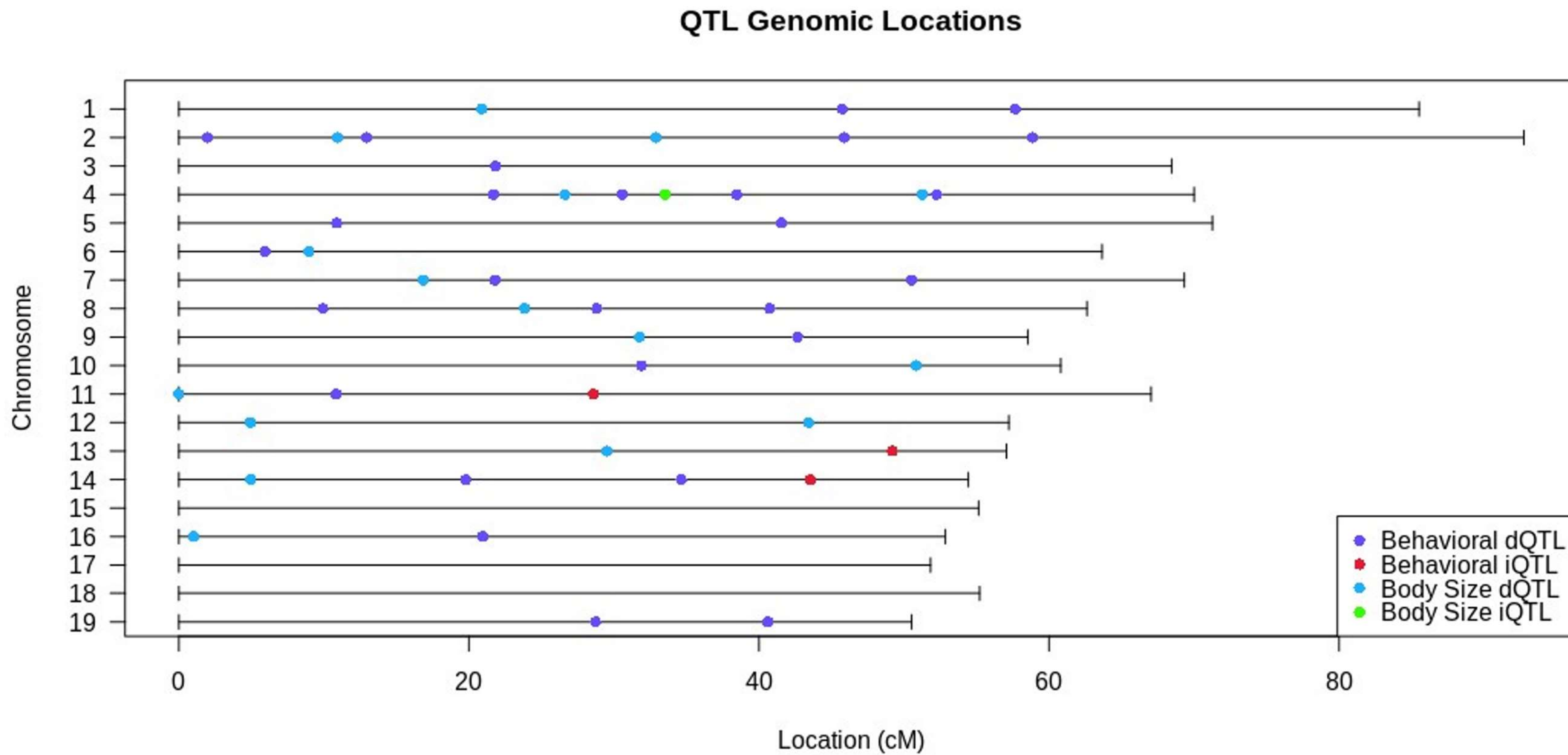


Figure 3.6. Phenotypic effects of iQTL in males and females. Phenotypes of each mouse are plotted against the genotype of the cagemate ("MM" = homozygous for mainland mouse alleles, "GM" = heterozygous, "GG" = homozygous for Gough Island mouse alleles). A. iQTL for cagemate body weight at 9 weeks of age. B. iQTL for time a cagemate spent in the light chamber in adult males. C. iQTL for time a cagemate spent in the light chamber, detected as a sex-by-genotype interaction. D. iQTL for distance a cagemate traveled in the first five minutes of the open field test in adult females.

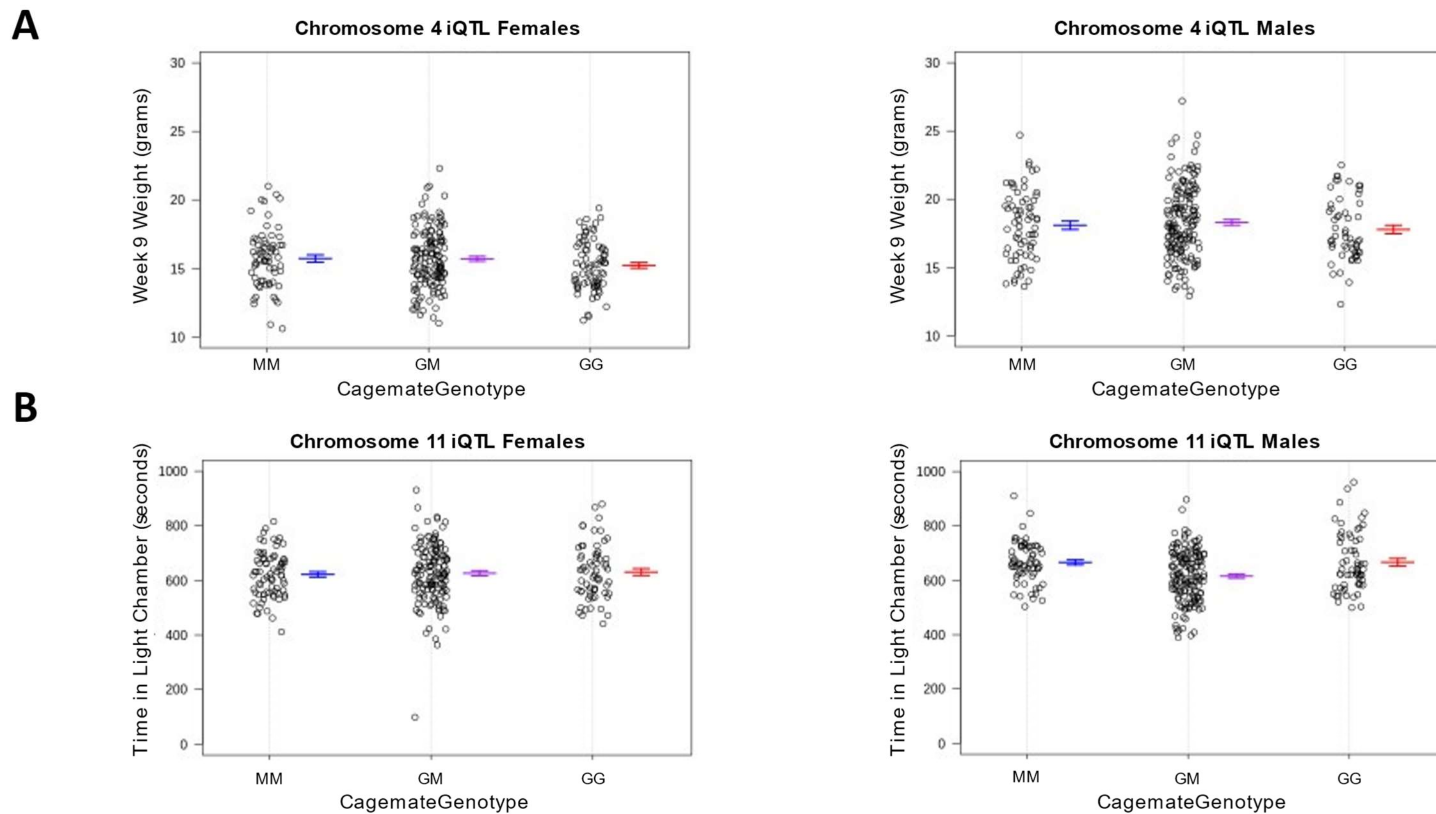


Figure 3.6 continued. Phenotypic effects of iQTL in males and females. Phenotypes of each mouse are plotted against the genotype of the cagemate ("MM" = homozygous for mainland mouse alleles, "GM" = heterozygous, "GG" = homozygous for Gough Island mouse alleles). A. iQTL for cagemate body weight at 9 weeks of age. B. iQTL for time a cagemate spent in the light chamber in adult males. C. iQTL for time a cagemate spent in the light chamber, detected as a sex-by-genotype interaction. D. iQTL for distance a cagemate traveled in the first five minutes of the open field test in adult females.

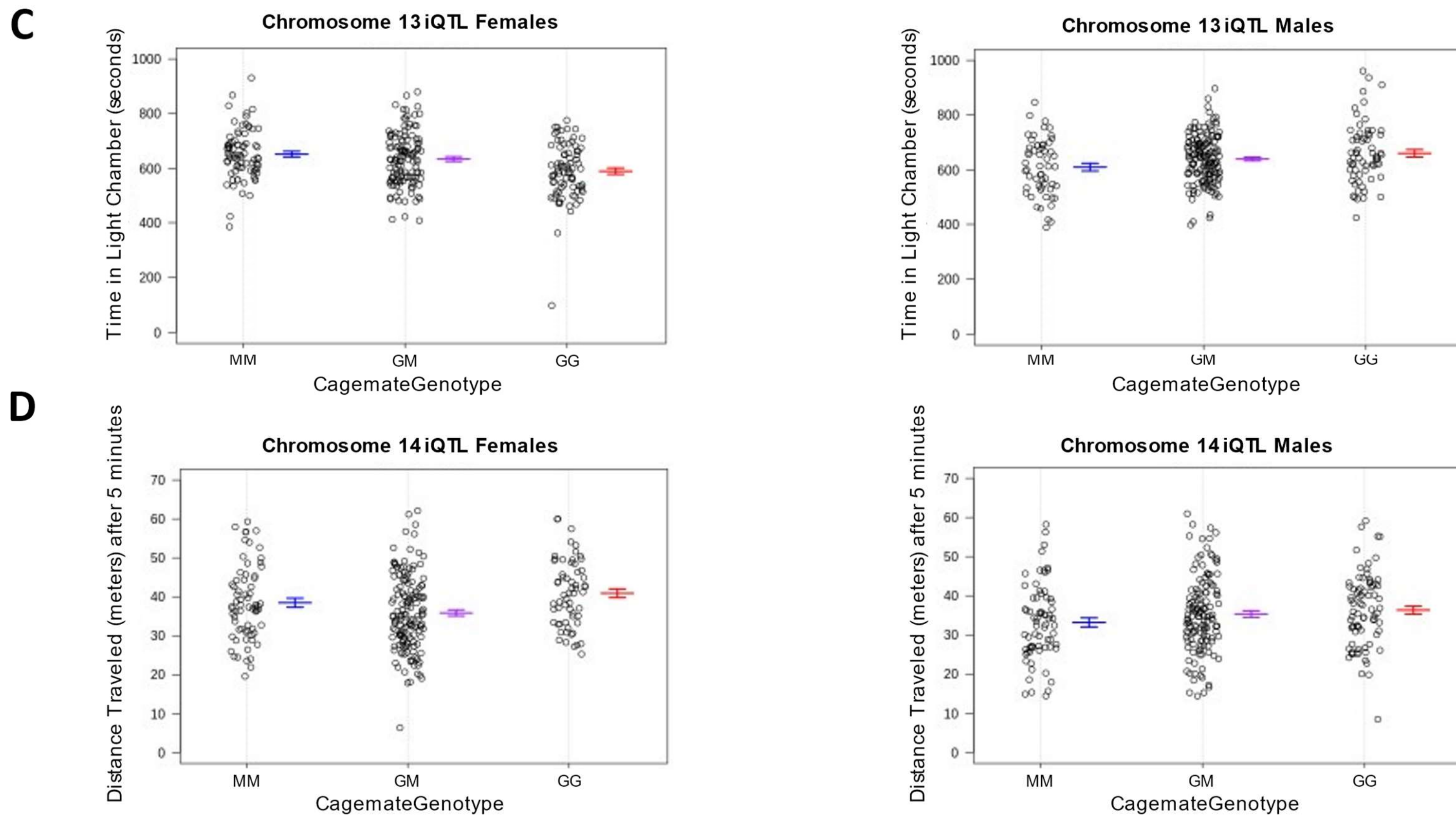


Figure 3.7. Percent of F2 variance explained by iQTL and direct QTL (dQTL) detected in the same cross. Data for dQTL underlying behavior are from Stratton *et al.* (2023). QTL detected for the same trait at multiple ages (body size, open field behaviors) were trimmed to the timepoint when the QTL explains the maximum amount of phenotypic variance.

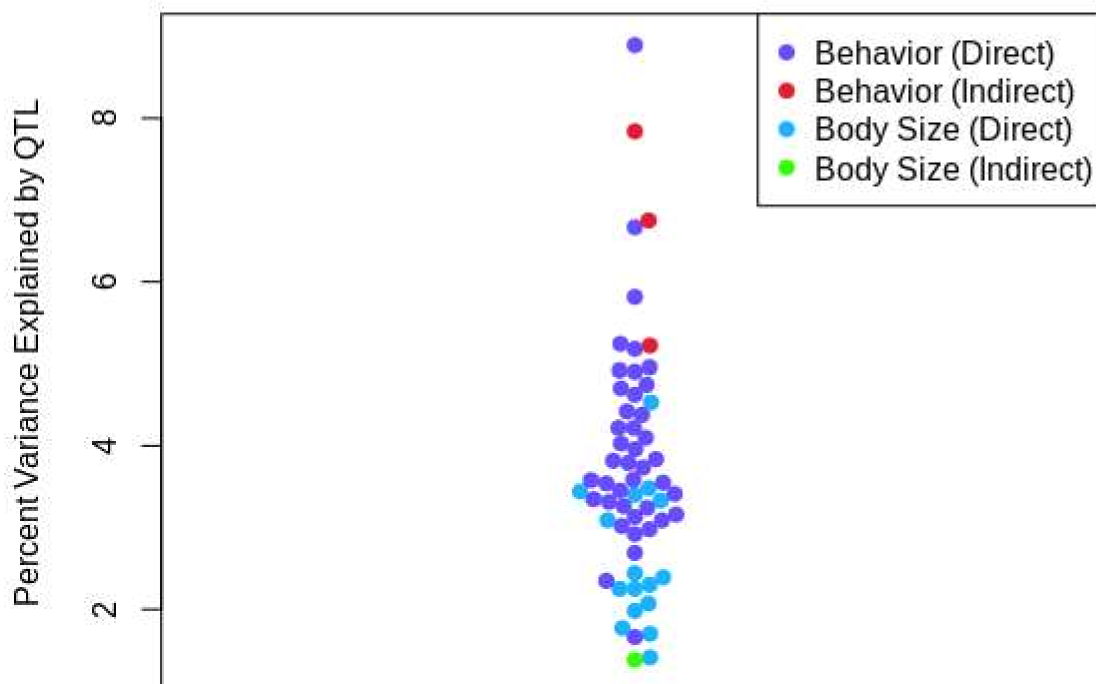


Table 3.1. Summary of iQTL. a. Position of the peak of the iQTL based on the GRCm39 assembly of the house mouse genome. b. Percent of F2 phenotypic variance explained by the sex-by-iQTL interaction effect was included for the iQTL on chromosome 13. c. Mean and standard error of genotypic class ("MM" = homozygous for mainland mouse alleles, "GM" = heterozygous, "GG" = homozygous for Gough Island mouse alleles).

Trait	Chromosome	LOD score	Peak Position (Mb)[a] [Lower Bound, Upper Bound]	Percent Variance Explained[b]	Additive Effect (Standard Error)	A/Sigma (SE)	Dominance Effect (SE)	D/A (SE)
Week 9 Body Weight	4	4.45	95.23 [48.65, 96.05]	1.38	-0.17 (0.11)	-0.063 (0.040)	0.623 (0.15)	-3.73 (2.5)
Time Spent in the Light Chamber (Males)	11	4.27	58.43 [51.83, 80.78]	6.75	1.16 (8.36)	0.012 (0.086)	-50.5 (11.29)	-43.53 (313.65)
Time Spent in the Light Chamber	13	5.17	109.66 [54.8, 115.41]	7.84	-29.28 (7.72)	-0.296 (0.078)	12.61 (11.69)	-0.43 (0.42)
Distance Traveled in the Open Field Minute 5 (Females)	14	4.23	106.44 [104.07, 119.69]	5.22	1.47 (0.73)	0.154 (0.077)	-3.98 (0.99)	-2.71 (1.5)

Table 3.1 continued. Summary of iQTL. a. Position of the peak of the iQTL based on the GRCm39 assembly of the house mouse genome. b. Percent of F2 phenotypic variance explained by the sex-by-iQTL interaction effect was included for the iQTL on chromosome 13. c. Mean and standard error of genotypic class ("MM" = homozygous for mainland mouse alleles, "GM" = heterozygous, "GG" = homozygous for Gough Island mouse alleles).

A*Sex Interaction Effect (SE)	D*Sex Interaction Effect (SE)	Mean (SE) of ♀ MM[c]	Mean (SE) of ♀ GM[c]	Mean (SE) of ♀ GG[c]	Mean (SE) of ♂ MM[c]	Mean (SE) of ♂ GM[c]	Mean (SE) of ♂ GG[c]
NA	NA	15.72 (0.25)	15.7 (0.17)	15.22 (0.24)	18.09 (0.31)	18.30 (0.20)	17.78 (0.34)
NA	NA	622.12 (11.67)	626.52 (8.52)	630.87 (12.55)	666.26 (11.58)	615.78 (7.57)	666.83 (11.85)
54.36 (11.7)	-5.7 (16.53)	652.26 (11.1)	634.18 (8.89)	589.66 (10.89)	610.19 (12.73)	640.13 (7.58)	660.59 (11.81)
NA	NA	38.48 (1.13)	35.85 (0.77)	40.91 (1.19)	33.18 (1.14)	35.33 (0.82)	36.36 (1.08)

Table 3.2. Candidate genes for iQTL. a. Number of sequence differences between Gough Island mice and mice from the mainland strain at sites that are conserved across placental mammals and located within 100kb of the gene.

Gene	Chromosome	Associated Trait	Number of Mutations in Conserved Sites[a]	Number of Protein Coding Changes	Associated Behaviors	Reference
Ambp	4	Week 9 Cagemate Weight	4	0	Reduced social exploration in KO strains	Goulding *et al.* 2019
Brinp1	4	Week 9 Cagemate Weight	5	0	Reduced social interaction in KO strains	Kobayashi *et al.* 2014
Dlg4	11	Cagemate Time in Light Chamber, Males	2	0	Reduced social exploration in KO strains	Coley and Gao 2019
					Increased aggression in KO and heterozygous KO strains	Winkler *et al.* 2018
Gria1	11	Cagemate Time in Light Chamber, Males	25	0	Reduced social interaction and aggression in knockout (KO) strains	Barkus *et al.* 2012
					Altered social interaction levels in KO strains depending on cell type	Kilonzo *et al.* 2022
					Reduced aggression in KO strains	Vekovischeva *et al.* 2004
					Shorter, altered forms of social interactions in KO strains	Wiedholz *et al.* 2008
Nlgn2	11	Cagemate Time in Light Chamber, Males	1	0	Reduction in social exploration when overexpressed	Hines *et al.* 2008
					Reduced social interaction in KO strains	Liang *et al.* 2015

Slc6a4	11	Cagemate Time in Light Chamber, Males	1	0	Increased social avoidance in heterozygous KO strains	Bartolomucci *et al.* 2010
					Reduced aggression in KO strains	Holmes *et al.* 2002
					Reduced attenuation of social exploration (impaired social recognition) in heterozygous KO strains	Page *et al.* 2009
					Reduced social interaction in KO and heterozygous KO strains	Tanaka *et al.* 2018
					Reduced social interaction in KO strains	Veenstra-VanderWeele *et al.* 2012
Cmya5	13	Cagemate Time in Light Chamber	25	30	Reduced social interaction in KO strains	Tsoupri *et al.* 2021
Gcnt4	13	Cagemate Time in Light Chamber	3	0	Increased social dominance (aggression) in KO strains	Stone *et al.* 2009
Ndufs4	13	Cagemate Time in Light Chamber	33	0	Greater docility in KO strains	Quintana *et al.* 2010
Rnf180	13	Cagemate Time in Light Chamber	11	0	Increased social exploration in KO strains	Kabayama *et al.* 2013

Acknowledgments

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Conclusion

This dissertation examines the genetic basis, both direct and indirect, of evolved behaviors in a population of house mice following colonization of a unique island environment. This work unites distinct subfields of behavioral genetics and evolutionary biology to provide a genetic portrait of behavioral evolution following drastic environmental shifts. I utilized a natural population of house mice from Gough Island (GI) with extreme phenotypic shifts across multiple trait categories that correspond to the unusual ecology of their environment. My work builds on previous genetic characterizations of morphological evolution in GI mice. I measured anxiety and exploration using established behavioral assays, which allows for direct comparisons to studies in laboratory mice. In Chapter 1, I show that GI mice have heritable increases in boldness and exploration which are absent when direct predator cues are present. I also find that behaviors are influenced by life stage (juvenile or adult), a parameter that is important in the development of animal personality but is rarely studied (Stamps and Groothuis 2010; Cabrera *et al.* 2021). In Chapter 2, I uncover many quantitative trait loci (QTL) underlying these behavioral shifts, several of which are context specific. I find that behaviors can be categorized into three genetically distinct groups: 1) anxiety, 2) exploration, and 3) defecation. Evolution within each group likely proceeded independently from the others. Each QTL may be expressed only in certain conditions. QTL may be unique to juvenile or adult life stages or impact behavior either before, after, or throughout habituation to a novel environment. QTL may also be restricted to certain environmental settings, such as the presence of a shelter. In Chapter 3, I reveal that the social environment impacts body size and behavior and has a heritable component. I identify four distinct QTL associated with phenotypes of cagemates, referred to as indirect QTL (iQTL). iQTL can have effects as large as direct QTL (dQTL) but are located in separate genomic regions and therefore are genetically uncorrelated. I also show that loci underlying morphological evolution (both direct and indirect) are largely separate from those underlying behavior. Importantly, iQTL were mapped using the same approaches and dataset used to detect dQTL, demonstrating an untapped potential of standard mapping studies.

A common theme throughout this dissertation is the importance of context in shaping behaviors or their underlying genetic factors. GI mice are among the boldest and most exploratory mice when compared to laboratory strains (Kumar 2021). However, they behave the same as mainland mice in the presence of fox urine (Chapter 1). Additionally, GI mice only show their extreme exploratory tendencies as adults. In contrast to morphology, where a proportion of QTL had pleiotropic effects on growth across the skeleton and overall body mass (Parmenter *et al.* 2016), distinct loci underlie different behavioral categories. This result is surprising given that boldness and exploration are predicted to covary across adaptive regimes, which suggests these traits are genetically correlated (Sih *et al.* 2004). My findings show that boldness and exploration can both evolve rapidly, even when they are controlled by distinct genetic loci. A possible facilitator of this coevolution may have been large amounts of standing variation in the mice that colonized GI. Standing genetic variation provides a head-start on the evolutionary process and is an important driver of adaptation (Barrett and Schluter 2008; Bomblies and Peichel 2022). Variation in boldness and exploratory tendencies may be reduced on the mainland through stabilizing selection. However, since many of the QTL I detected only contribute effects under certain conditions, there may have been cryptic variation for these traits that was revealed after the mice colonized the island.

The context specificity of evolved behaviors and underlying QTL is one of several limitations in this thesis. I discussed in Chapters 1 and 2 how behavioral differences between GI mice and mainland mice could have occurred along either evolutionary branch. While I listed reasons that advocate for island-associated causes of the QTL I discovered, behavioral profiling in additional mainland populations would bolster confidence in the conclusions I've drawn. Additionally, while my sample size is several hundred individuals larger than most genetic crosses using natural populations, my ability to detect QTL with small effects is still limited. It is likely that I missed many QTL, leading to overestimated effects of QTL I discovered. Unidentified QTL may also show pleiotropy or linkage across different phenotypes,

changing the observed independence of evolution among traits. Despite these limitations, the depth of phenotypic characterization I conducted provides new perspectives on the genetics of behavioral evolution.

A unique feature of this dissertation was the characterization of indirect genetic effects (IGE) underlying evolved behaviors. The intercross design and QTL mapping approach I used are common. However, the role of cagemates on focal phenotypes is traditionally ignored. I found that iQTL can have effects as large as dQTL and can be mapped using standard methods. Since GI mice lived in much higher densities than mainland mice (Rowe-Rowe and Crafford, 1992), the effects of the iQTL I identified were likely enhanced in the natural population due to the greater number of conspecifics each mouse interacted with. One advantage of mapping iQTL over variance-based approaches for IGE estimation is the ability to examine genetic properties of individual loci. Dominance was found to be an important characteristic of iQTL, with one iQTL being purely underdominant. The allele frequency at this locus in the founder population of GI could have had a substantial impact on the evolutionary trajectory for boldness in GI mice. Extreme allele frequencies at the causative site in either direction would have mitigated the effects of this iQTL due to the low number of heterozygotes.

Future directions

This dissertation leaves many unanswered questions regarding the evolution of behavior in GI mice. Due to their commensal relationship with humans, house mice have spread all over the world (Phifer-Rixey and Nachman 2015; Morgan *et al.* 2022) and colonized multiple islands (Berry *et al.* 1979; Berry *et al.* 1987; Miller and Miller 1995; Ganem 1998; Moro *et al.* 2003; Mathias *et al.* 2004; Berry 2009; Jones *et al.* 2011; Renaud *et al.* 2013). However, the genetic characterization of island-associated behaviors I conducted is rare. As such, it is uncertain if the genetic architecture I uncovered is the rule

or the exception. Genetic characterization of other insular populations is needed to build a general understanding of how insular behaviors evolve. Given the impactful role of IGEs in our population, I encourage other studies to search for iQTL to allow for comparisons. No additional data were needed to discover iQTL in our intercross population. While I used traditional mapping methods to successfully uncover iQTL, additional methods could improve upon my analysis. For example, cagemate permutations reveal that the iQTL I identified may be spurious. Orthologous approaches, such as the development of congenic strains to test the effects of iQTL, would provide needed validation of the associations I detected. I also used genotype data at peak markers underlying dQTL to account for their effects, but a scan that jointly considers the genomes of both focal individuals and cagemates may improve power and refine estimates of QTL positions and effects. Epistatic interactions between direct and indirect loci are also currently unexplored.

Results from Chapter 1 revealing that behavioral differences are context dependent raise the question of how GI mice and mainland mice would behave in conditions mimicking the natural environment of GI. While challenging to pursue, natural simulation experiments can reveal adaptations that are cryptic in laboratory conditions and improve our understanding of the adaptive process. For example, Colella, *et al.* (2021) showed that desert-adapted cactus mice portray metabolic optimization only in diurnally variable conditions approximating the natural fluctuations in temperature and humidity from their native habitat. The most likely driver of reduced anxiety in GI mice is the lack of predators, a variable that is identical between the island and laboratory environments. This suggests that the extreme behaviors of GI mice in the open field and light/dark box tests are maintained on the island. However, the connection between behavioral shifts and improved fitness in GI mice is unclear. Increased boldness on the island is predicted to be adaptive by increasing resource acquisition with no cost of predation (Pyke 1984; Stephens and Krebs 1986; Brown and Kotler 2004), a situation that highly skews the growth-mortality tradeoff which balances earlier versus later reproduction (Promislow and

Harvey 1990; Stamps 2007). A common method of evaluating foraging tendencies in simulated natural environments is giving-up density experiments (Brown 1988; Brown and Kotler 2004; McMahon *et al.* 2018). These tests force subjects to forage for food in arenas which can mimic natural conditions under the discretion of the experimenter. Using these experiments, we could determine if behavioral shifts in GI mice lead to improved foraging success and observe the corresponding effects on growth.

The true history of behavioral evolution in GI mice is unknowable until causal variants are identified. I listed strong candidate genes underlying dQTL and iQTL impacting behavior in F2 mice. Importantly, many of these genes are also implicated in models of human behavioral disorders. Insights from further characterization of the QTL I discovered could therefore reveal genetic mechanisms associated with psychiatric illnesses. However, each QTL region contains tens to hundreds of genes and each gene may have tens to hundreds of sequence changes between GI mice and mainland mice. Until causal variants are identified, we cannot be sure which genes were drivers of behavioral evolution and where the natural variation in behavioral shifts originated. Others have used laboratory strains of mice to successfully fine-map QTL for fear and seizure susceptibility (Parker *et al.* 2013; Winawer *et al.* 2014). Molecular dissection of QTL conferring local adaptations in three-spine sticklebacks has been successful in identifying causal mutations underlying changes in plate morphology (Colosimo *et al.* 2004; Colosimo *et al.* 2005; O'Brown *et al.* 2015), lateral line patterning (Wark *et al.* 2012; Mills *et al.* 2014), schooling behavior (Greenwood *et al.* 2013; Greenwood *et al.* 2016), development of the pelvis (Shapiro *et al.* 2004; Chan *et al.* 2010), number of teeth (Cleves *et al.* 2014; Miller *et al.* 2014; Ellis *et al.* 2015), and pigmentation (Miller *et al.* 2007). Perhaps most notably, detailed molecular dissection identified the circadian clock components in fruit flies (Bargiello and Young 1984; Reddy *et al.* 1984; Zehring *et al.* 1984) and house mice (King *et al.* 1997).

Identification of causal mutations is one of two important steps towards a fundamental understanding of the behavioral evolution of GI mice. The other key step is determining the underlying

phenotypes and molecular mechanisms which the causal mutations confer (Flint 2019). I hypothesized *a priori* that the mechanisms underlying behavioral differences operate primarily in the brain, but the nature of those mechanisms remains a mystery. Fear and anxiety are controlled by multiple regions of the brain, though the amygdala plays an especially important role (Adolphs 2013). Transcriptomic analysis can be used to measure the activity of the amygdala and other brain regions integrally involved in fear and memory (such as the hypothalamus and prefrontal cortex). Differences in expression patterns between GI mice and mainland mice could highlight shifts in pathways that correlate with evolved behaviors. A complementary approach involves measuring the connection between the hypothalamic-pituitary-adrenal (HPA) axis, a central system in the mammalian stress response (Smith and Vale 2006; Kolber *et al.* 2008), and evolved behaviors. In Chapter 1, I used corticosterone as a readout of the HPA axis to discover that both GI mice and mainland mice are highly stressed after exposure to a predator cue. In Chapter 3, I describe a correlation between corticosterone concentration and anxiety-like behavior in adult female cagemates. However, whether the increased boldness of GI mice in the open field corresponds to a reduced activation of the stress response is unknown. Additionally, the level of chronic stress in the home cage environment remains a mystery. Repeated measures of corticosterone concentration during cohabitation with mice from different genetic backgrounds would help determine potential mechanisms of social influences on anxiety. Elucidating the activity of the stress response across environmental and experimental conditions could reveal connections between internal physiological status and evolved behaviors.

The mechanisms governing iQTL effects are similarly mysterious. I discussed in Chapter 3 how behaviors changing the number and kinds of social interactions between cagemates are likely to be driving the iQTL I discovered. To explain correlations in body size between cagemates, I hypothesized that increased huddling could improve thermoregulation and promote growth. To explain social impacts on anxiety, I hypothesized that agonistic behaviors between cagemates could alter levels of

boldness in subsequent behavioral assays. Both hypotheses can be tested by monitoring social pairs within the home cage. Advanced metabolic cage technology can gather behavioral and metabolic data from inhabitants constantly without human intervention (Reynolds *et al.* 2021). These systems could identify instances when cagemates are in close contact and have elevated metabolic rates. After validation from video recordings, correlates of aggressive bouts (such as increased respiration) can be used to measure agonistic behavior automatically and efficiently in many pairs of mice. Such home-cage experiments may be used to determine the nature of interactions between inbred strains of GI mice, mainland mice, and pairs of both strains. These tests can be complemented by aggression assays in novel environments with unfamiliar conspecifics. Reduced aggression is expected to evolve on islands as a response to increased population densities (Adler and Levins 1994) but these trends in natural populations have so far been non-heritable (Baier and Hoekstra 2019). Conversely, I have identified heritable variants that impact social phenotypes without knowing their connection to aggression. Knowing the rates of aggression in GI mice and mainland mice against familiar and unfamiliar conspecifics is a key step in understanding the nature of iQTL and their impacts on the evolution of GI mice.

A holistic understanding of the adaptive process requires a broad view of organismal complexity. I began this thesis by discussing the need for investigations that consider many different traits in locally adapted populations. The morphology of GI mice has been extensively examined at the genetic level (Gray *et al.* 2015; Parmenter *et al.* 2016; Nolte *et al.* 2020; Parmenter *et al.* 2022). This dissertation describes the evolution of behavioral extremes in GI mice to add another axis of organismal complexity. I hope that the number and kinds of investigations characterizing GI mouse evolution continue to expand, providing a wealth of knowledge rarely obtained from a single natural population.

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Appendix A

Supplementary Material for Chapter 1

Supplementary Table 1.1. Raw measurements and associated metadata for each mouse used in this study. Supplementary Table 1.1 may be found online with the original article (https://link.springer.com/article/10.1007/s00265-021-03003-6#Sec17)

Supplementary Table 1.2. Summaries of linear model results for F1s in the open field. The third column shows transformations performed on the dependent variable. The fourth column shows significant independent variables among Sex, Cross Direction, and Age. When none of these predictors were significant, the overall mean is presented as the Intercept Estimate.

Experi-ment	Trait	Transfor-mation	Independent Variables	Intercept Estimate	Cross Direction (Mainland Mother) Effect	Cross Direction p-value	Sex (Male) Effect	Sex p-value	Age (Adult) Effect	Age p-value
Open Field	Distance Traveled (meters)	None	Sex, Cross Direction	159.765	16.123	0.021	-15.437	0.031	-	-
	Time Spent Mobile (seconds)	None	Sex	606.42	-	-	-74.44	0.003	-	-
	Time in Center (seconds)	None	None	101.0735	-	-	-	-	-	-
	Number of Fecal Boli	None	Age	7.3276	-	-	-	-	1.4224	0.003

Supplementary Files 1.1 – 1.9. Computational scripts used in video analysis (Supplementary Files 1.1-1.9) are available online at

https://github.com/jeredastrat/gough_mouse_behavior/tree/main/Stratton_et_al_2021/

Appendix B

Supplementary Material for Chapter 2

Supplementary File 2.1.

Mouse Strains and Husbandry

In 2009, GI mice were live-caught and shipped to the University of Wisconsin School of Veterinary Medicine Charmany Instructional Facility, where a breeding colony was established (Gray *et al.* 2015). GI mice were then bred for over 20 generations of brother-sister mating to establish an inbred line. Mainland mice belong to the WSB/EiJ inbred strain (JAX stock #001145) founded from breeding pairs caught in Maryland and were maintained in the same colony as GI mice for the same period of time. GI mice and house mice from the eastern coast of North America are likely descended from Western European mice (Gray *et al.* 2014; Agwamba and Nachman 2022), though the geographic locations of source populations are unknown. Mice were housed in micro-isolator cages with corn cob substrate (1/8th inch; The Andersons Lab Bedding), with *ad libitum* access to food (Envigo 2020X Teklad Global Diet) and water. Breeders were provided with a higher fat chow (Envigo 2019 Teklad Global Diet) and a red mouse igloo (Bio Serv). All cages were provided with nesting material and irradiated sunflower seeds (Envigo). Cage changes occurred every 6-8 days or, in the case of a new litter, 10 days after parturition. The colony was kept in a temperature-controlled room (20-23°C) under a 12-hour light/dark cycle.

Mice used for behavioral testing were weaned at 21 days after parturition or at 20 days in the case of double litters. Mice were housed with one littermate of the same sex to reduce behavioral effects of within-cage hierarchies (Horii *et al.* 2017). All mice were weighed to the nearest tenth of a gram during cage changeouts and after the last behavioral assay (i.e., weekly from ages 4-9 weeks old +/- 1 day).

Phenotypic data from 638 F2s among 19 different breeding pairs were generated over the

course of this project. Twenty-five mice were removed following quality control screening for genotypes (see "Genotyping"). For various reasons (e.g., conflicts with the experimenter's schedule, mice escaping the colony, etc.), some F2s received only one or two behavioral tests, creating imbalances in sample sizes among behavioral assays.

Behavioral Assays

All behavioral assays were conducted in a room separate from the main colony. All tests were conducted during the light phase of the light/dark cycle under white, fluorescent light. Females were scored for stage of estrous cycle according to Caligioni (2009) on the same day as testing, beginning with the second open field test. The order of subjects tested within litters each day was randomized. Each test began by bringing the subjects' cage into the room for a 30-minute acclimation period. The experimenter remained in the room out of sight of the subjects throughout the acclimation and testing periods except for transfer to and from the arena. The relevant arena for each test was placed in the center of the room next to a movable cart where a computer and video recording hardware were located. The arena was cleaned with 70% ethanol after each test. For the open field test at least five minutes was allowed for the ethanol to evaporate before testing a new subject. For the light/dark test the floor panels were removed and shaken to evaporate the ethanol instead of using a five-minute waiting period. All tests were video recorded using Debut Video Capture Software v 5.33 at default settings with Focus set to 0. A Logitech HD Pro Webcam C920 was positioned directly above the center of the arena. Two videos were taken before each test to assist in video analysis: an "empty" video containing a short recording of the empty arena, and a calibration video containing a short recording of the arena with specific positions marked. The "empty" video was used to align all images of the test recording. The calibration video was used to define coordinates of regions of interest and to convert pixels to millimeters. Subsequent subsections provide additional details of experimental design for each

test.

Open Field Test

The open field (58cm W x 58cm D x 58cm H; Figure 1B) was constructed from expanded, white PVC (Grainger Industrial Supply). Lighting in the room was set so that the center of the open field measured 300 +/- 5 lux. A calibration video was taken using a poster board on the bottom of the arena with the center and each corner (1 inch from both edges) marked by circles drawn with black marker. Subjects were initially placed in the center of the arena facing away from the movable cart. The video recording software was started, and the subject was allowed to freely explore the arena. After 30 minutes of uninterrupted exploration, the subject was returned to its home cage and the number of fecal boli in the arena was counted, a commonly used readout of anxiety originally validated in the rat (Hall 1934).

Light/Dark Test

A mouse-sized place-preference chamber (68.6cm W x 20.3cm D x 38.1cm H; San Diego Instruments; Figure 1C) was used for a light/dark test. One half of the arena had a black floor and was exteriorly covered with black static cling film. The opposite chamber had a white floor and was exteriorly covered (interiorly covered in Stratton *et al.* (2021)) with white static cling film except for the lid to allow video observation. The chamber divider was positioned 4 cm above the floor to allow free access between both chambers and was covered in black static cling film on the side facing the light chamber. Lighting was set so that the center of the light chamber measured 300 +/- 5 lux. A calibration video was taken using a separate floor panel placed on top of the floor in the white chamber with the center and each corner (1.27 cm from both edges) marked by circles drawn with black marker. Subjects were initially placed in the center of the light chamber facing the entrance to the dark chamber. The

video recording software was started and the subject was allowed to freely explore the arena. After 30 minutes of uninterrupted exploration, the subject was transferred to a glass jar, anesthetized using isoflurane, and decapitated preceding blood and organ collection. The number of fecal boli in each chamber was then counted.

Analysis of Videos

To minimize observer bias, the processing of behavioral videos was automated and blind with respect to mouse ancestry. Videos were translated into x and y coordinates of the subject for each frame of the video using the following pipeline. First, the raw videos were converted using ffmpeg via the following command:

```
ffmpeg -i inputFileName.avi -pix_fmt nv12 -f avi -vcodec rawvideo convertedFileName.avi
```

The converted video file was run through an ImageJ script associated with Mousemove (Samson *et al.* 2015), which we modified to label frames deleted due to inability to maintain the framerate or frames when no mouse was detected. Light/dark videos were manually cropped to view only the light chamber to speed processing and improve background subtraction. Deleted frames and frames when multiple objects were detected were reincorporated using the position of flanking frames. Calibration videos were run through the same process to obtain positions of known locations in the arena. The tracking software tends to erroneously detect multiple objects. The trajectory files in such instances were manually edited to remove objects that never moved (i.e., non-mouse objects).

Combined trajectory files were then analyzed to measure a variety of different traits depending on the test (see below). Pixels were translated to mm using known distances between objects in the

calibration trajectory file. A movement threshold was set for each subject using the method of Shoji (2016). Output for each trait was recorded for every minute of the test for use in function-valued trait analysis.

The center of the open field was defined as a circle with a 26.59 cm radius (14.5 cm radius in Stratton *et al.* (2021)) which stretches to 1 inch from the edge of the arena. Distance traveled was the sum of all positional changes above the movement threshold in the combined trajectory file.

Time spent past two different thresholds in the light chamber were recorded: 1) 1.27 cm (0.5-inch) from the dark chamber, and 2) midpoint of the light chamber. The number of crosses past these thresholds was also recorded.

Genotyping

F2 carcasses were stored at -20°C following euthanasia. At a later date, carcasses were thawed and liver tissue was collected and stored at -20°C. Later, all liver samples were submitted to Neogen for genotyping on the Giga Mouse Universal Genotyping Array (https://www.neogen.com/categories/genotyping-arrays/gigamuga/). The array contains over 143,000 markers selected to provide maximal information for studies utilizing the Collaborative Cross (Aylor *et al.* 2011; Threadgill and Churchill 2012), and Diversity Outbred Cross (Svenson *et al.* 2012) with additional markers selected to capture diversity in wild mouse populations.

Quality Control Checks

Several controls were used to identify and remove samples and markers showing patterns that suggested errors. All parents (F1s) and grandparents (GI and mainland) of the 638 F2s were included in the genotyping to assess residual heterozygosity within inbred strains and assist in quality control

checks. The grandparents of the F2s were submitted in duplicate to assess the genotyping error rate. Rates of missingness and signal intensity among plates were analyzed to assess plate performance.

Genotype calls on the sex chromosomes and mitochondria were checked to ensure that each sample's genotype met expectations based on the recorded sex and pedigree (i.e., cross direction) of the mouse. Four mice with mismatched phenotypic and genotypic sex predictions were removed. An additional 20 samples were removed based on the following criteria: 1) over 10% missing data among informative markers, or 2) over 2% missing data among informative markers and abnormally high signal intensity variance among all markers.

Marker level checks were performed on the remaining dataset of 614 mice. Markers with missing data in over 30 samples or heterozygous calls in under 270 samples were removed. Evidence for segregation distortion was evaluated using the geno.table() function in R/qtl. Following the method outlined in Broman and Sen (2009), markers with a p-value less than 1e-7 were removed. A genetic map was built using the Carter-Falconer map function assuming a 0.1% genotyping error rate. The first marker on chromosome 18 was subsequently removed as it appeared unlinked to the rest of the chromosome. Genotyping error LOD scores were calculated using the calc.errorlod() function. Calls with error LOD scores of 5 or higher were replaced with NAs. The number of inferred crossovers for each mouse was calculated. One additional sample was removed at this step due to a high number of inferred crossovers.

The final dataset included 613 F2s with genotype information at 31,681 informative markers across all autosomes and the X chromosome.

Single Trait QTL Mapping

Single trait (hereafter "ST") analyses were conducted using the value of each behavioral trait

from the culmination of the assay. To begin, genotype probabilities for each individual were calculated using the calc.genoprob() function in R/qtl assuming a 0.1% genotyping error rate. Genotype probabilities were calculated at every marker and at a set of additional pseudomarkers to ensure probabilities were present at every 0.2cM. Single-QTL analysis was conducted using Haley – Knott regression (Haley and Knott 1992). To determine which covariates to include in the QTL scan, environmental and pedigree-related predictors were evaluated using linear models for each trait. Independent variables were included as additive covariates when deemed significant by an additional sum-of-squares test comparing models that included or excluded the variable. To examine the presence of sex-specific QTL we conducted scans with sex as an interactive covariate for traits where sex was an additive covariate (Time in Center Juvenile, Distance Traveled Juvenile, Distance Traveled Adult, Fecal Boli Adult, Light Chamber Entrances, Center Crosses, and Fecal Boli Total). Thresholds for QTL significance were calculated using a permutation test (Churchill and Doerge 1994). QTL with LOD scores in the top 5% among 1,000 permutations (cutoff ranging from 3.93 to 4.19) were deemed significant for autosomes. 17,836 permutations were collected for the X chromosome to determine a separate threshold following the methodology in Broman *et al.* (2006). QTL identified by single QTL scans were used as a starting model for a multiple QTL model search algorithm implemented in stepwiseqtl(). Briefly, a penalized LOD score for each model is calculated using the following equation:

$$\mathrm{pLOD}(\gamma) = \mathrm{LOD}(\gamma) - T|\gamma|$$

where γ is the model being tested, $|\gamma|$ is the number of QTL in the model and T is a penalty set to the 5% significance threshold (separate for autosomes and the X chromosome) in the single QTL scan. The algorithm proceeds in a forward manner up to a model of 15 QTL choosing the model with the highest pLOD score at each step. The algorithm then proceeds backwards down to a model with no QTL. The model with the highest pLOD score among all models visited is selected as the best-fit model. Occasionally, phenotypic outliers can create two tightly linked, false-positive QTL if the outlier has a rare

recombination event between two markers. When tightly linked QTL were present in the best-fit model they were removed if fewer than 25 individuals had recombination events between them.

Function-valued Trait Mapping

Function-valued trait QTL mapping was conducted using r.funqtl (Kwak *et al.* 2015). To begin, values for each behavior at each minute of the test (1-30) were smoothed using B-splines. Then, the collection of functional principal components (fPCs) that explained over 99% of the variance in the data were extracted. The fPCs were then fit to the same covariates used in ST analyses and residuals were extracted for QTL mapping. Since ST analyses produced no evidence of X-linked QTL, the X chromosome was excluded from function-valued trait mapping to improve computational speed and compliance with the software. Three different mapping approaches were used: 1) "HK" multivariate mapping following Knott and Haley (2000), 2) "SL" mapping which takes the average LOD score across scans of each fPC individually, and 3) "ML" mapping which takes the maximum LOD score across scans of each fPC individually. Permutations to establish significance thresholds were conducted in a similar manner to the ST analyses using the scanoneM() function with the appropriate mapping method specified. QTL deemed significant at the 5% level were used as a starting point in a multiple-QTL model search algorithm akin to ST analyses, proceeding forward to a model of 12 QTL and down to a model of 0 QTL. The best-fit model was scrutinized for false positives caused by outliers with rare recombination events.

Scans for Epistasis

Scans for epistasis were conducted using two approaches. First, we used the scantwo() function in R/qtl with a set of pseudomarkers at fixed 0.5cM intervals and the same additive covariates used in additive only QTL scans. The X chromosome was excluded from this analysis to avoid elevated interaction LOD scores (Broman and Sen 2009). All autosomal locations were allowed to interact.

Significance for epistasis was based on the interaction LOD score among 1,000 permutations. Second, we used the scanonevar() function to search for QTL affecting the variance of each trait (vQTL). Significance was based on the LOD score among 1,000 permutations using the scanonevar.perm() function.

Estimation of QTL Effects

Additive and dominance effects of QTL were estimated using the fitqtl() function in R/qtl. QTL for each trait, regardless of which method identified them, were combined into a single QTL object along with covariates used in the QTL mapping. Effects for all QTL were estimated simultaneously at each minute of the assay. To improve comparisons among QTL, the additive effect of each locus was standardized by the phenotypic standard deviation at each timepoint.

Candidate Gene Nomination

The 1.5 LOD confidence interval for each QTL was used to extract an initial list of genes from the Mouse Genome Informatics (MGI) database (http://www.informatics.jax.org/marker/). We searched for "protein-coding genes" using the term "behavior" under "Phenotypes/Diseases". All genes from these initial lists are found in Supplementary Table 3. We then extracted mutations between our two inbred strains within these genes using the UCSC Variant Annotation Integrator Tool (http://genome.ucsc.edu/cgi-bin/hgVai) applied to genome sequences of GI mice (Nolte *et al.* 2020) and WSB mice (Keane *et al.* 2011). We used two different searches to collect mutations of interest. First, we filtered for "CDS – nonsynonymous", "exon loss caused by deletion", and "splice site or splice region" within "Functional roles". Then, we filtered for all functional roles except "Intergenic" while filtering for elements conserved across placental mammals. Next, we extracted expression data using the MGI Gene Expression Database (http://www.informatics.jax.org/gxd). We noted if a gene was found to be

expressed within the amygdala, hippocampus, or prefrontal cortex, three brain regions known to be associated with anxiety and exploration. Genes with at least 1 mutation of interest which were expressed in at least one of the three brain regions of interest were further scrutinized for associations to behavior in the literature.

Evidence for Selection

Posterior probabilities of positive selection in 5kb windows across the GI mouse genome were calculated in Payseur and Jing (2020). These authors used the SWIF(r) (Sugden *et al.* 2018) machine learning approach to compute posterior probabilities of directional selection from a variety of summary statistics describing patterns of variation among 14 wild-caught GI mice and their mainland counterparts. We extracted the maximum posterior probability within each candidate gene region to evaluate evidence for selection acting on behavioral candidate genes.

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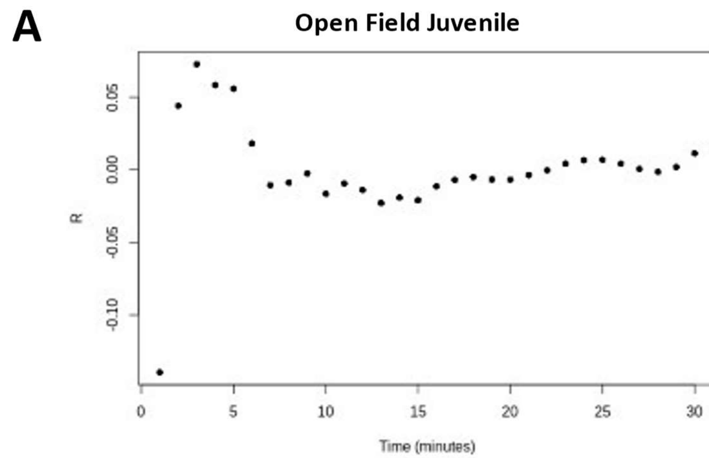
Supplementary File 2.2. Summary of QTL at each minute of the behavioral assay. Supplementary File 2.2 is available online at

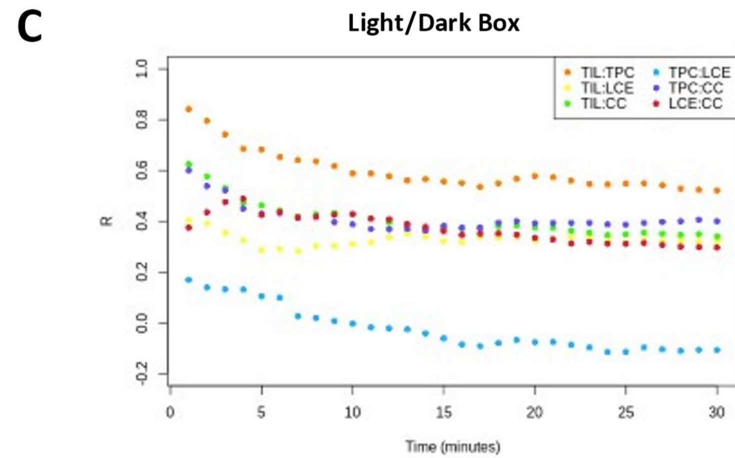
https://github.com/jeredastrat/gough_mouse_behavior/tree/main/Stratton_et_al_2023

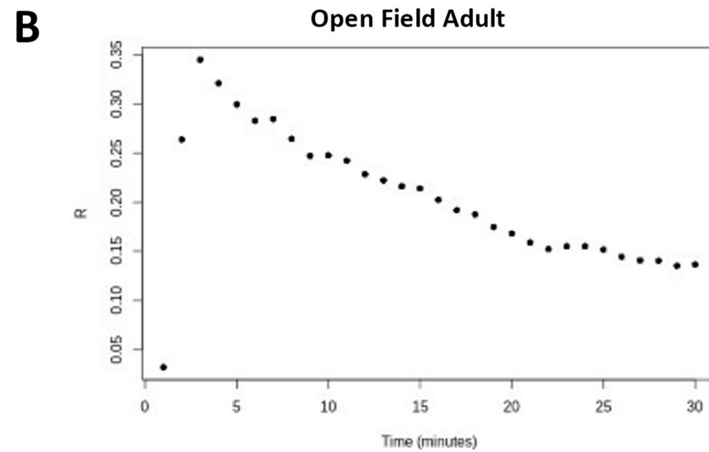
Supplementary Files 2.3 – 2.9. Computational scripts used in video analysis (Supplementary Files 2.3-2.9) are available online at

https://github.com/jeredastrat/gough_mouse_behavior/tree/main/Stratton_et_al_2023

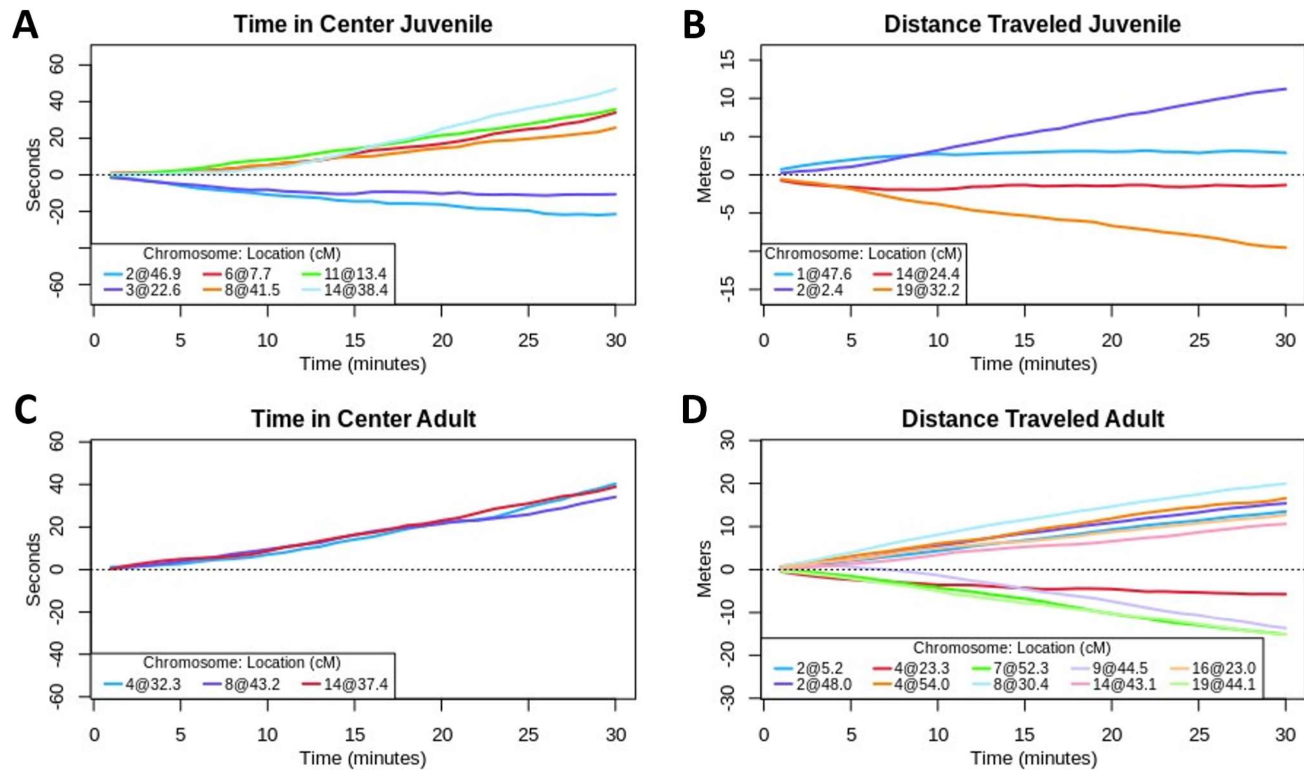
Supplementary Figure 2.1. Correlations between traits across F2s during the course of an assay. A. Correlation between time in center and distance traveled during the open field test in juveniles. B. Correlation between time in center and distance traveled during the open field test in adults. C. Correlations between traits during the light/dark box test ("TIL" = "time in light chamber"; "TPC" = "time past center"; "LCE" = "light chamber entrances"; "CC" = "center crosses").



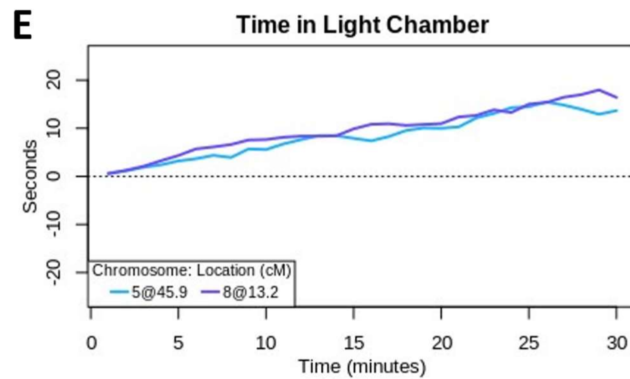


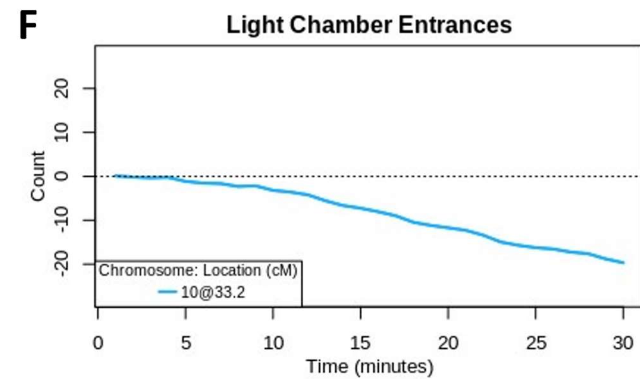


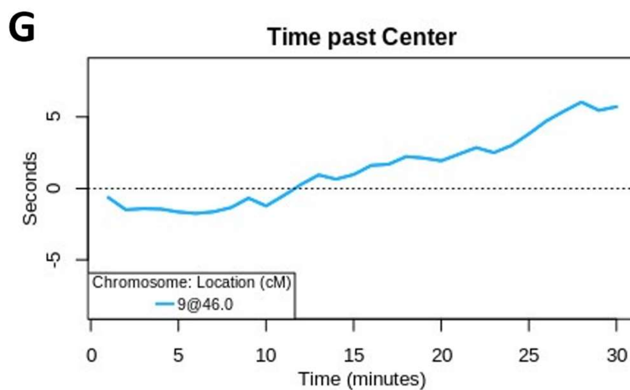
Supplementary Figure 2.2. Additive effects of QTL. Additive effects of QTL were jointly estimated at every minute of the assay (1 to 30). A. Time spent in the center of the open field as juveniles. B. Distance traveled in the open field as juveniles. C. Time spent in the center of the open field as adults. D. Distance traveled in the open field as adults. E. Time spent in the light chamber of the light/dark box. F. Number of entrances to the light chamber. G. Time spent past the center of the light/dark box. H. Number of crosses past the center of the light/dark box.

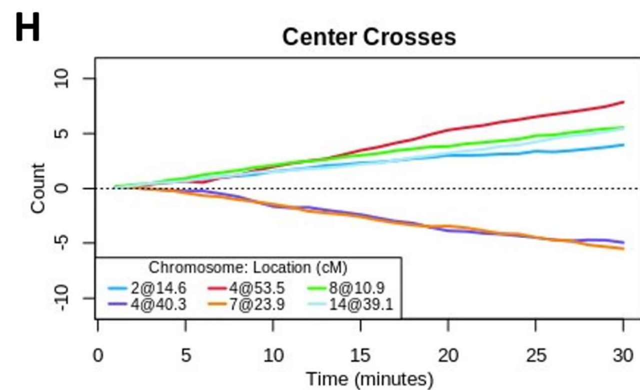


Supplementary Figure 2.2 continued. Additive effects of QTL. Additive effects of QTL were jointly estimated at every minute of the assay (1 to 30). A. Time spent in the center of the open field as juveniles. B. Distance traveled in the open field as juveniles. C. Time spent in the center of the open field as adults. D. Distance traveled in the open field as adults. E. Time spent in the light chamber of the light/dark box. F. Number of entrances to the light chamber. G. Time spent past the center of the light/dark box. H. Number of crosses past the center of the light/dark box.



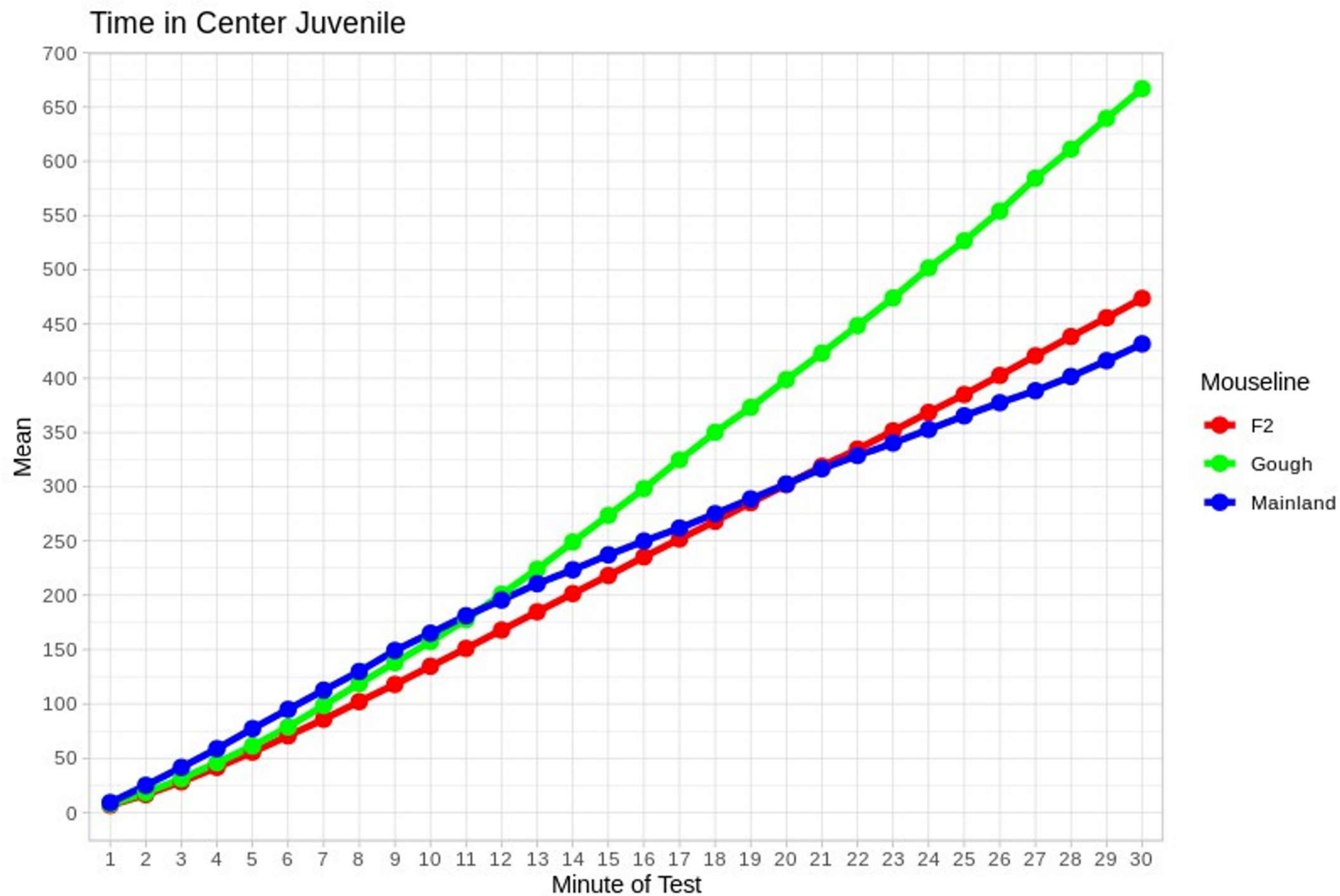






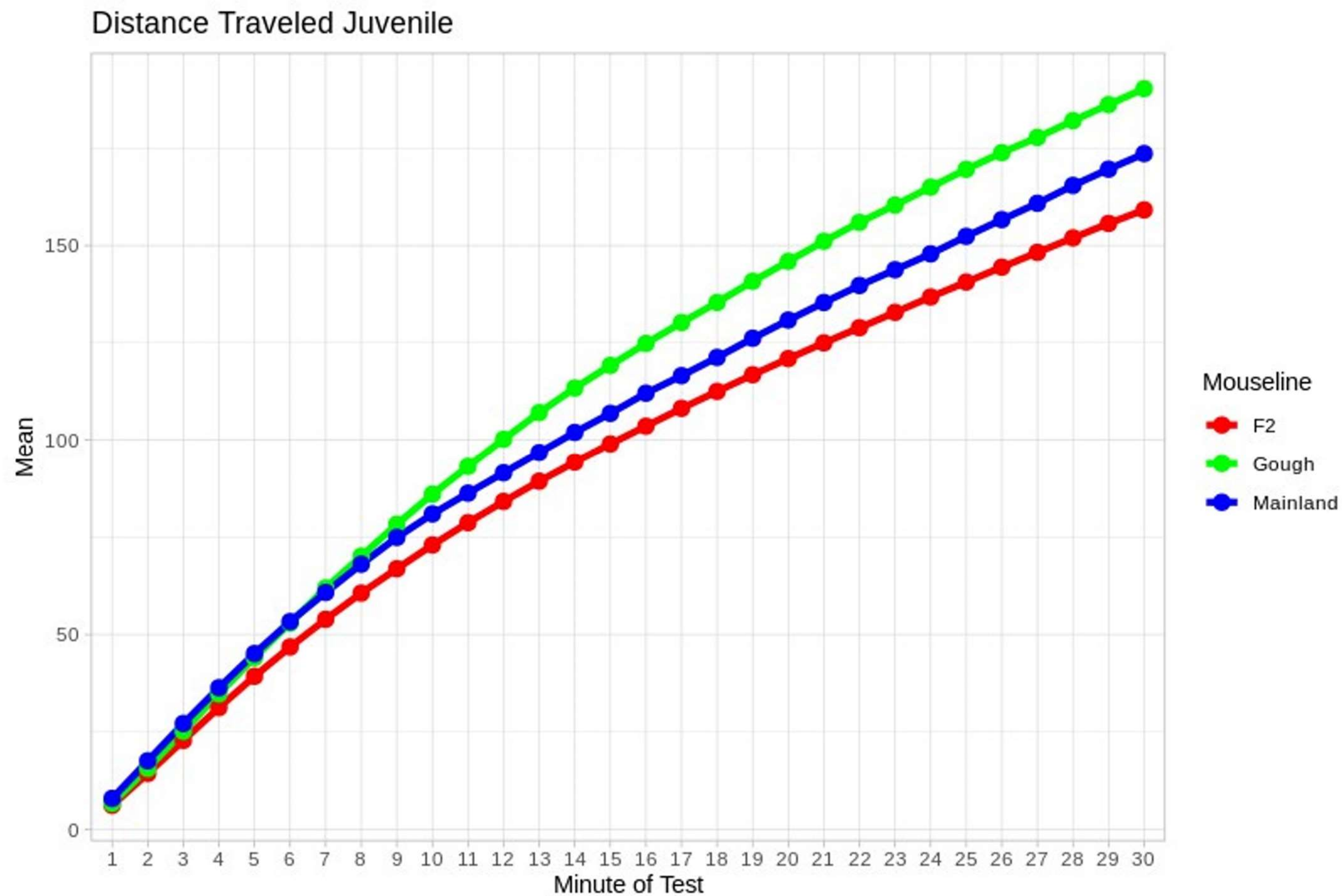
Supplementary Figure 2.3. Mean of behavioral measurements within parental strains and F2s at each timepoint of an assay. A. Time spent in the center of the open field as juveniles. B. Distance traveled in the open field as juveniles. C. Time spent in the center of the open field as adults. D. Distance traveled in the open field as adults. E. Time spent in the light chamber of the light/dark box. F. Number of entrances to the light chamber. G. Time spent past the center of the light/dark box. H. Number of crosses past the center of the light/dark box.

A

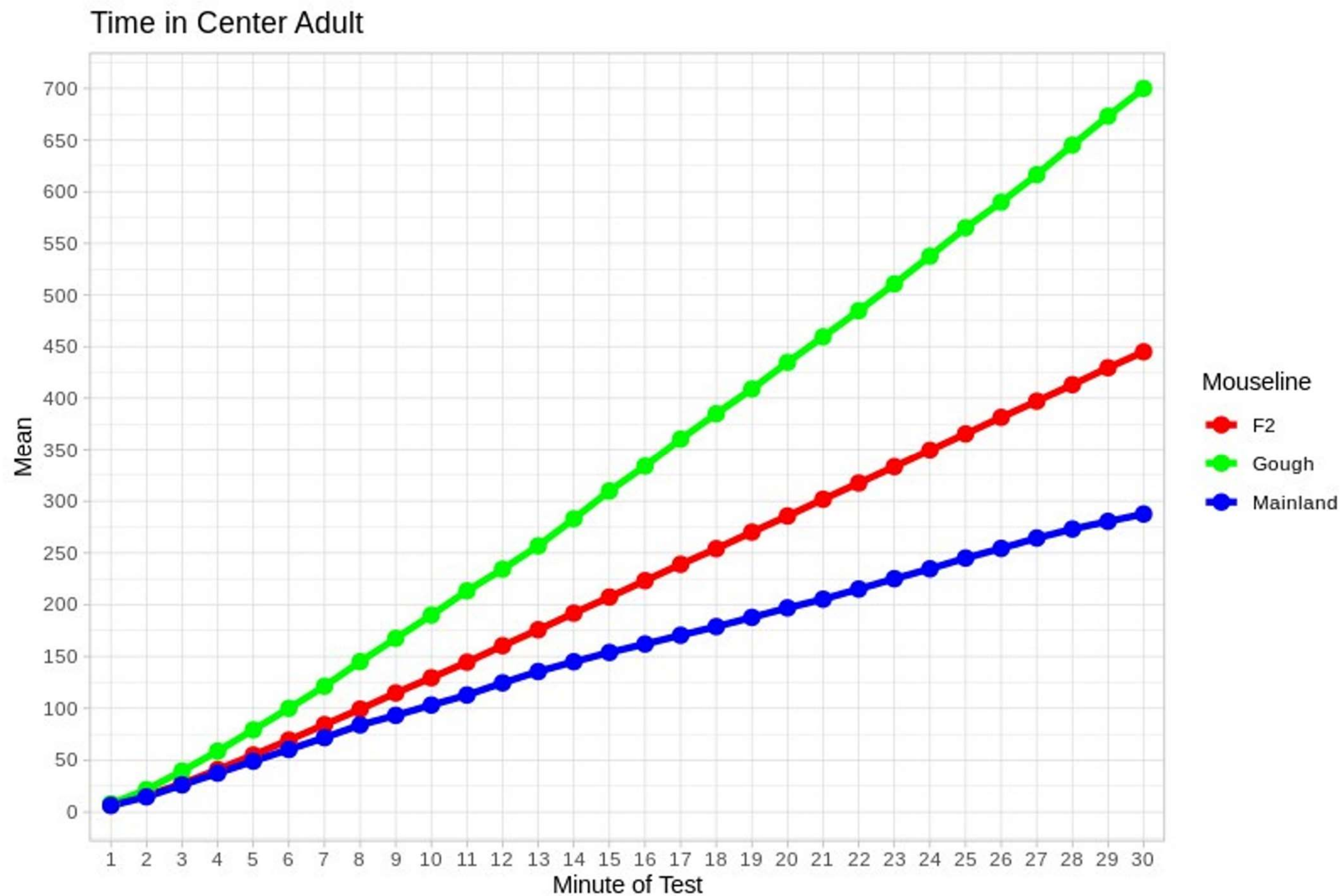


Supplementary Figure 2.3 continued. Mean of behavioral measurements within parental strains and F2s at each timepoint of an assay. A. Time spent in the center of the open field as juveniles. B. Distance traveled in the open field as juveniles. C. Time spent in the center of the open field as adults. D. Distance traveled in the open field as adults. E. Time spent in the light chamber of the light/dark box. F. Number of entrances to the light chamber. G. Time spent past the center of the light/dark box. H. Number of crosses past the center of the light/dark box.

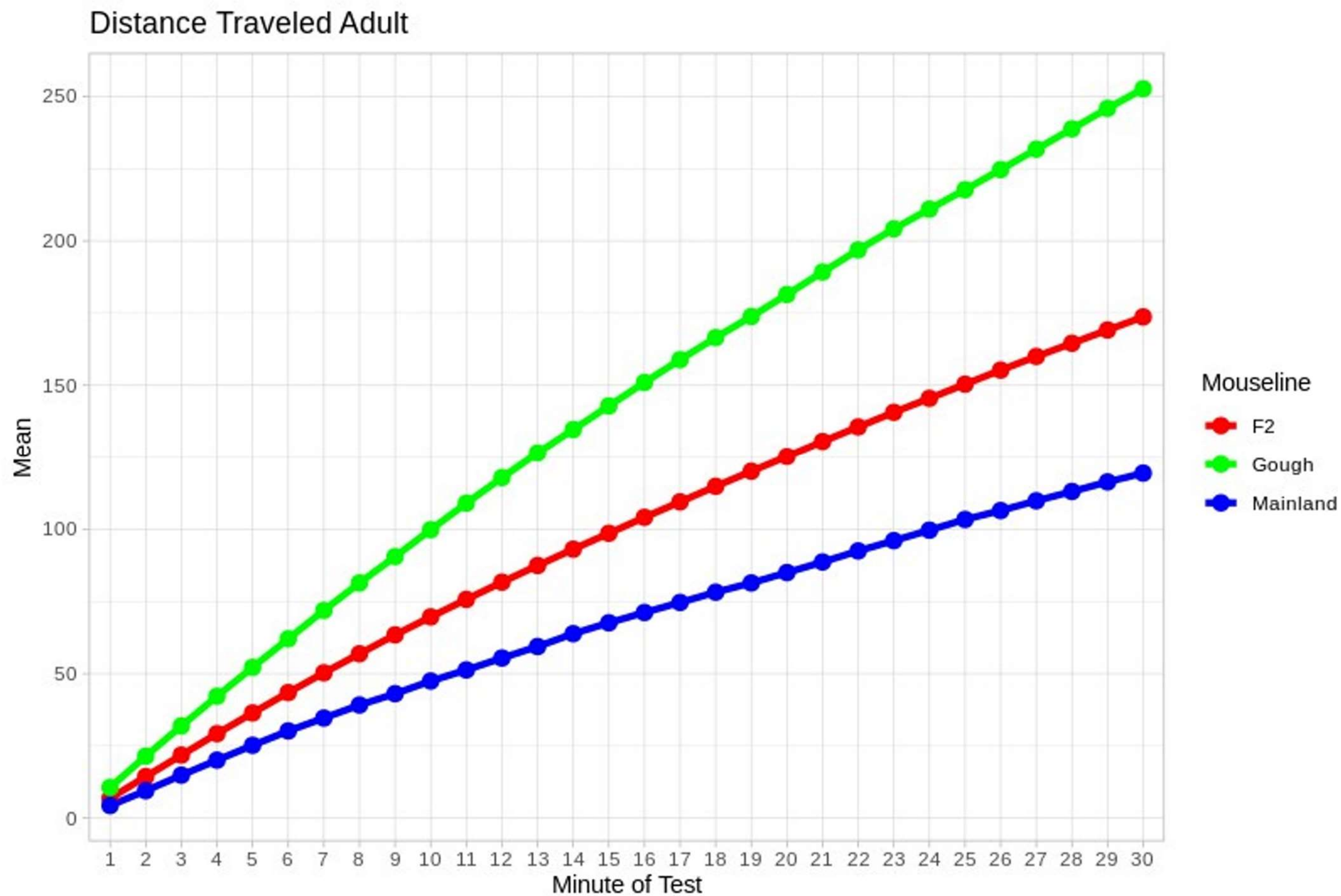
B



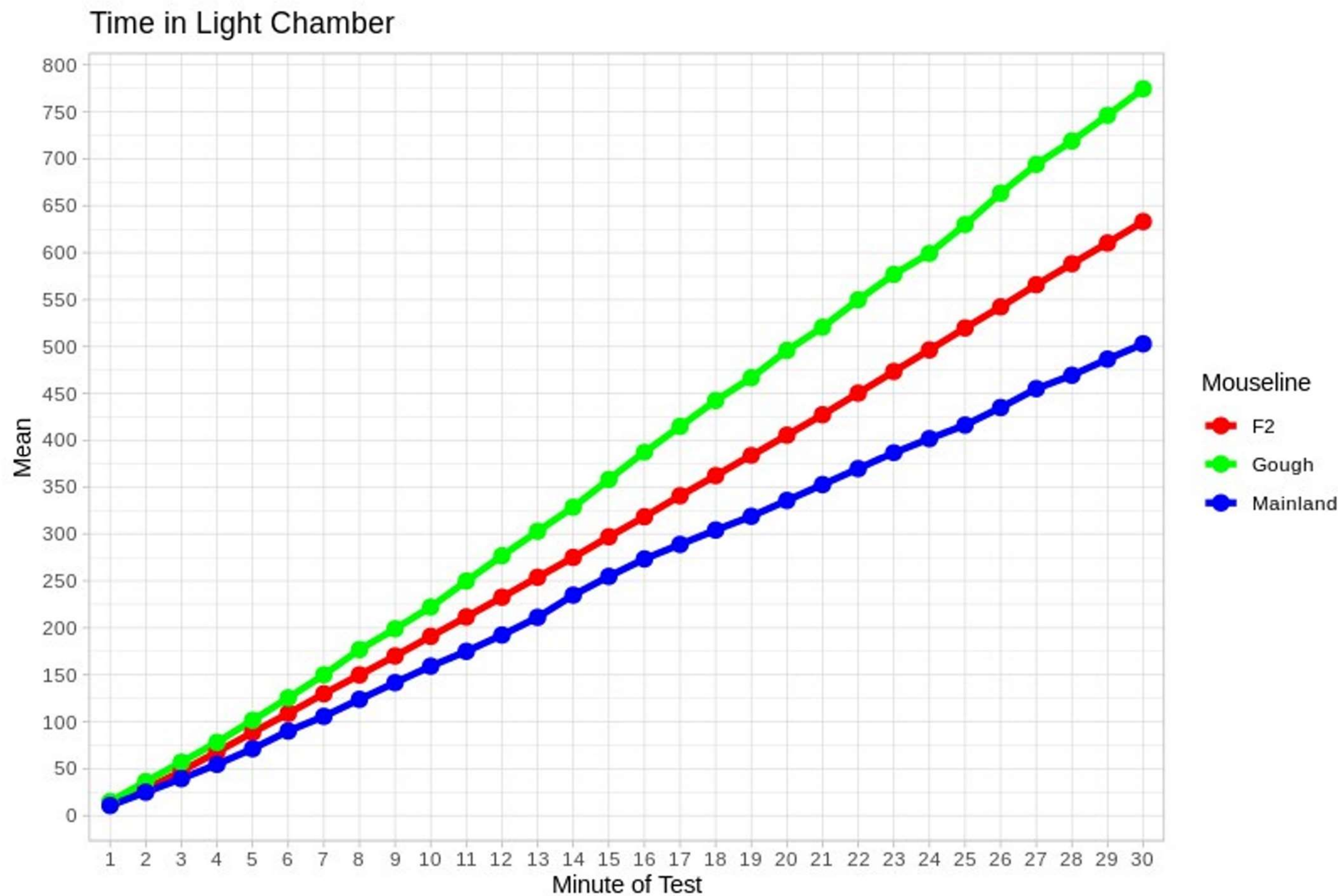
Supplementary Figure 2.3 continued. Mean of behavioral measurements within parental strains and F2s at each timepoint of an assay. A. Time spent in the center of the open field as juveniles. B. Distance traveled in the open field as juveniles. C. Time spent in the center of the open field as adults. D. Distance traveled in the open field as adults. E. Time spent in the light chamber of the light/dark box. F. Number of entrances to the light chamber. G. Time spent past the center of the light/dark box. H. Number of crosses past the center of the light/dark box.



Supplementary Figure 2.3 continued. Mean of behavioral measurements within parental strains and F2s at each timepoint of an assay. A. Time spent in the center of the open field as juveniles. B. Distance traveled in the open field as juveniles. C. Time spent in the center of the open field as adults. D. Distance traveled in the open field as adults. E. Time spent in the light chamber of the light/dark box. F. Number of entrances to the light chamber. G. Time spent past the center of the light/dark box. H. Number of crosses past the center of the light/dark box.

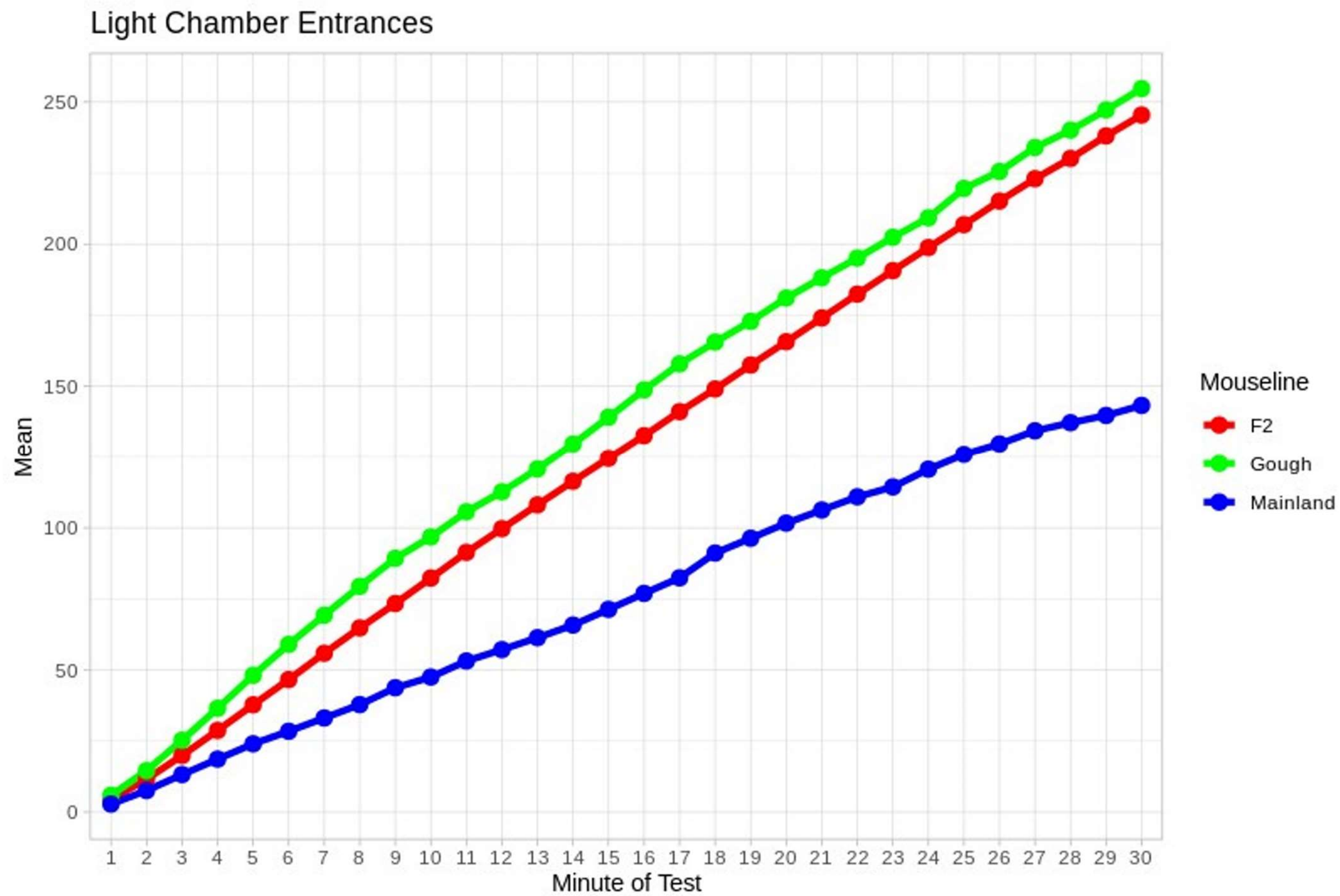


Supplementary Figure 2.3 continued. Mean of behavioral measurements within parental strains and F2s at each timepoint of an assay. A. Time spent in the center of the open field as juveniles. B. Distance traveled in the open field as juveniles. C. Time spent in the center of the open field as adults. D. Distance traveled in the open field as adults. E. Time spent in the light chamber of the light/dark box. F. Number of entrances to the light chamber. G. Time spent past the center of the light/dark box. H. Number of crosses past the center of the light/dark box.



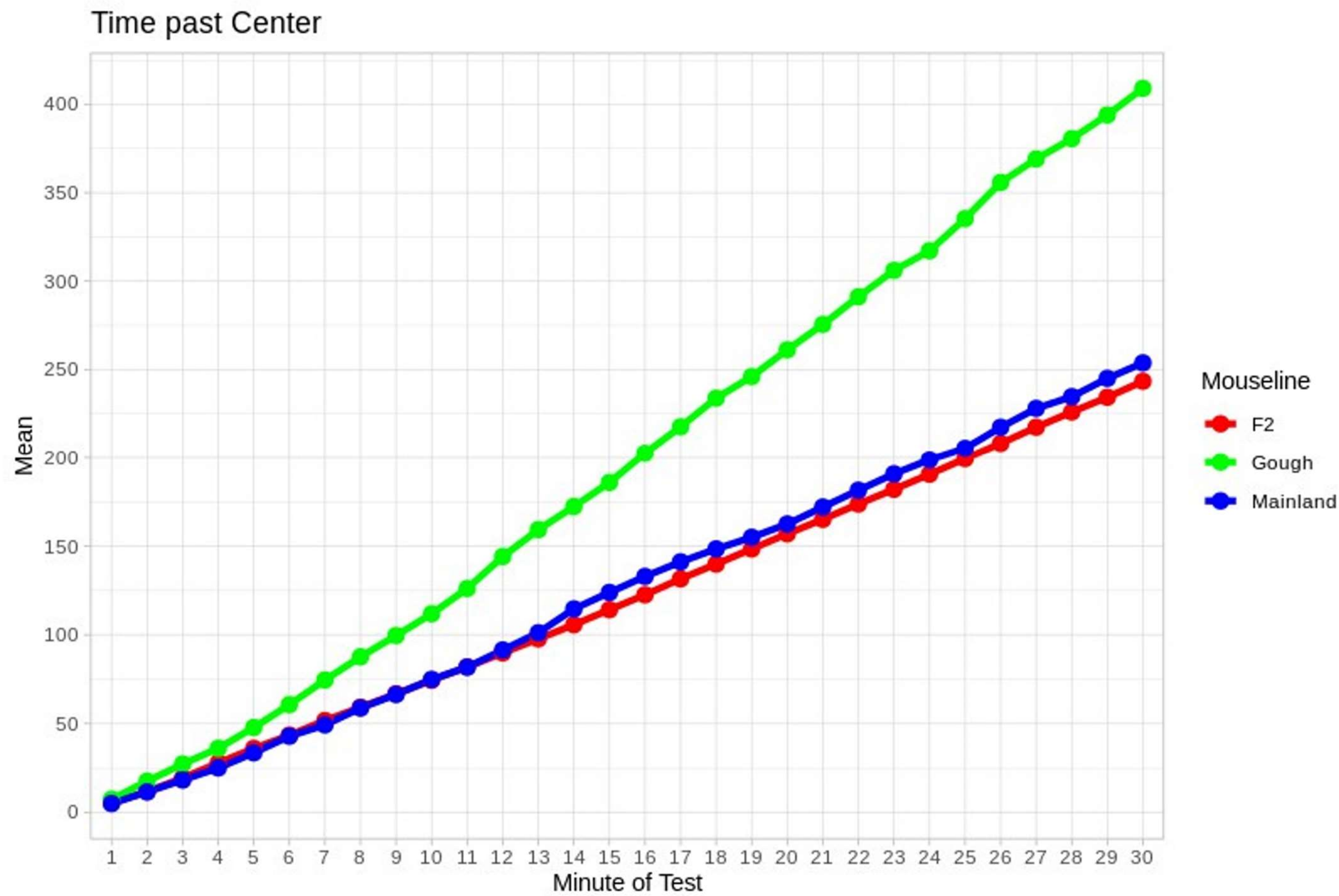
Supplementary Figure 2.3 continued. Mean of behavioral measurements within parental strains and F2s at each timepoint of an assay. A. Time spent in the center of the open field as juveniles. B. Distance traveled in the open field as juveniles. C. Time spent in the center of the open field as adults. D. Distance traveled in the open field as adults. E. Time spent in the light chamber of the light/dark box. F. Number of entrances to the light chamber. G. Time spent past the center of the light/dark box. H. Number of crosses past the center of the light/dark box.

F

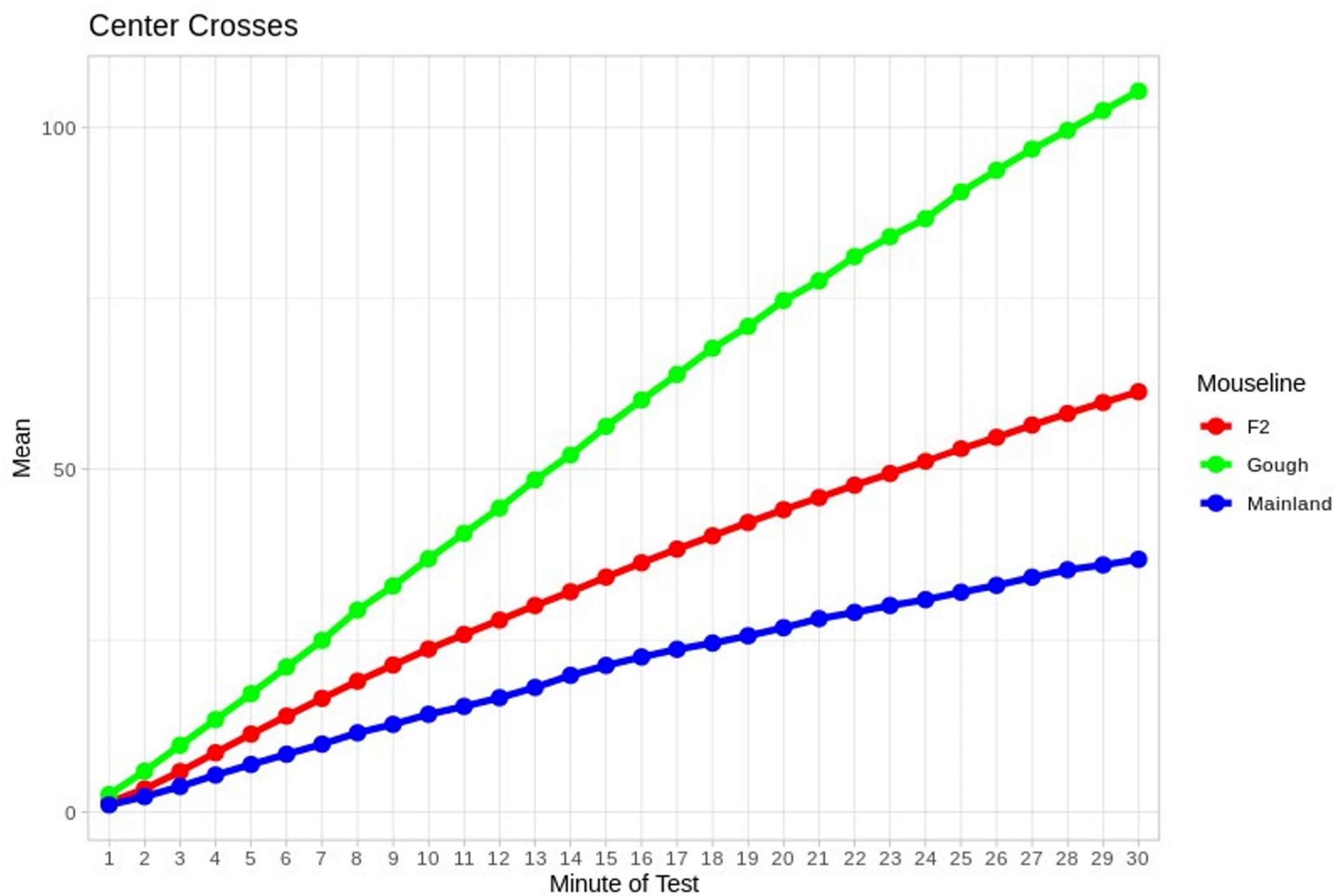


Supplementary Figure 2.3 continued. Mean of behavioral measurements within parental strains and F2s at each timepoint of an assay. A. Time spent in the center of the open field as juveniles. B. Distance traveled in the open field as juveniles. C. Time spent in the center of the open field as adults. D. Distance traveled in the open field as adults. E. Time spent in the light chamber of the light/dark box. F. Number of entrances to the light chamber. G. Time spent past the center of the light/dark box. H. Number of crosses past the center of the light/dark box.

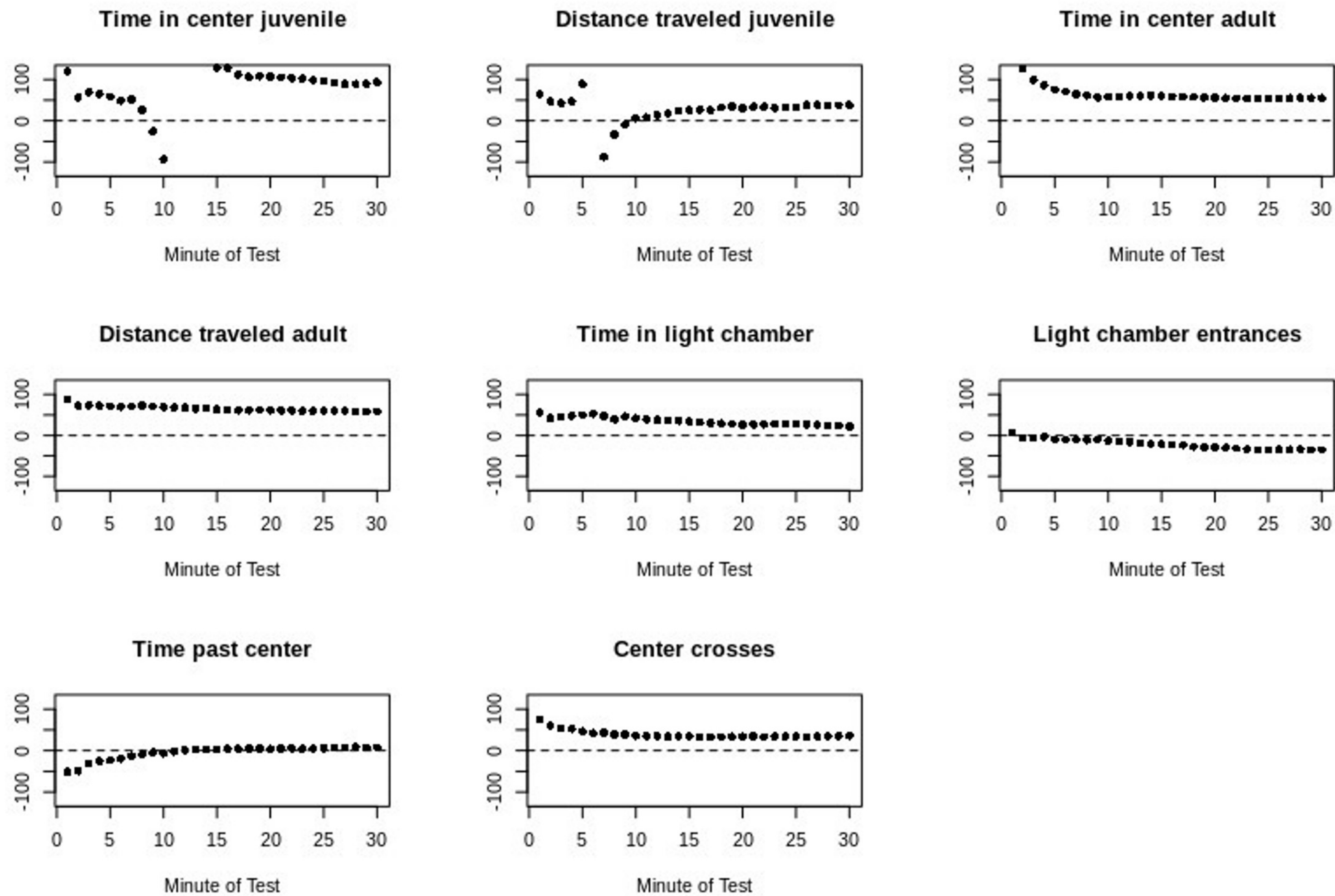
G



Supplementary Figure 2.3 continued. Mean of behavioral measurements within parental strains and F2s at each timepoint of an assay. A. Time spent in the center of the open field as juveniles. B. Distance traveled in the open field as juveniles. C. Time spent in the center of the open field as adults. D. Distance traveled in the open field as adults. E. Time spent in the light chamber of the light/dark box. F. Number of entrances to the light chamber. G. Time spent past the center of the light/dark box. H. Number of crosses past the center of the light/dark box.



Supplementary Figure 2.4. Percent difference between parental means explained by QTL over the course of each assay. Percent difference explained = $(\sum_{i=1}^{n}$ (2*A_i)/($\hat{G}$ - $\hat{M}$))*100, where A_i is the additive effect of QTL *i*, $\hat{G}$ is the mean of the Gough Island strain and $\hat{M}$ is the mean of the mainland strain.



Supplementary Table 2.1. List of covariates used for each trait in QTL analyses. Humidity, time of day, estrous, and temperature are each specific to the time when each assay was conducted. Time of day was recorded in zeitgeber hours where 0 is the start of the light cycle.

Trait	Sex	Parturition Number	Litter Size	Mother	Humidity	Time of Day	Estrous	Tempera-ture
Body Weight	X	X	X	X				
Time in Center Juvenile	X			X	X			
Distance Traveled Juvenile	X	X				X		
Fecal Boli Juvenile				X				
Time in Center Adult			X	X	X	X		
Distance Traveled Adult	X					X	X	
Fecal Boli Adult	X				X	X		
Time in Light Chamber			X					X
Light Chamber Entrances	X				X	X		
Time past Center								
Center Crosses	X			X		X	X	
Fecal Boli Total	X		X		X	X		

Supplementary Table 2.2. Raw phenotypes and metadata for the 613 F2s used in QTL analyses. Litter.ID denotes the F1 pairing that generated each mouse and was used to construct the "Mother" covariate. Supplementary Table 2.2 is available online at

https://github.com/jeredastrat/gough_mouse_behavior/tree/main/Stratton_et_al_2023

Supplementary Table 2.3. Summary of QTL models for loci identified by single trait mapping. The sum of additive effects and percent variance explained by all QTL in the model is at the bottom of each trait.

Assay	Age	Trait	Chromosome	LOD score	Percent Variance Explained	Additive Effect (A)	Std. Err. (A)
Open Field	Juvenile	Time in Center	6	5.78	3.38	32.13	6.84
			8	5.51	3.23	25.14	7.09
			11	5.43	3.17	35.38	7.21
			14	10.04	5.97	47.46	7.27
		Sum	-	-	15.76	140.11	-
		Distance Traveled	2	6.31	4.26	11.13	2.08
			19	5.05	3.4	-9.74	2.03
		Sum	-	-	7.66	1.39	-
		Fecal Boli	5	4.4	3.09	-0.54	0.14
	Adult	Time in Center	4	7.17	4.41	40.3	7.48
			8	4.74	2.89	34.11	7.42
			14	7.57	4.66	38.89	7.8
		Sum	-	-	11.96	113.31	-
		Distance Traveled	2	4.42	2.52	14.08	3.16
			2	4.83	2.76	15.28	3.28
			4	4.17	2.38	13.89	3.25
			7	4.84	2.76	-14.16	3.03
			8	9.65	5.62	19.95	3.13
			9	4.93	2.82	-12.86	3.05
			16	3.26	1.85	12.26	3.21
			19	4.07	2.32	-13.79	3.26
		Sum	-	-	23.02	34.66	-
		Fecal Boli	2	6.23	3.82	-0.7	0.15
			3	7.51	4.62	0.89	0.16
			5	8.38	5.18	-1.74	0.49
			5	5.58	3.41	1.09	0.48

		Sum	-	-	17.03	-0.46	-
Light/Dark Box		Light Chamber Entrances	10	5.09	3.79	-19.67	4.18
		Center Crosses	1	6.36	3.58	7.58	1.46
			2	3.53	1.96	3.86	1.01
			4	4.21	2.35	-4.51	1.47
			4	6.45	3.63	7.38	1.47
			7	6.75	3.8	-5.36	1
			8	7.16	4.04	5.47	0.98
			14	7.47	4.22	5.68	1.04
		Sum	-	-	23.58	20.09	-
		Fecal Boli	2	6.9	4.75	-0.99	0.18
			5	6.15	4.22	-0.72	0.19
		Sum	-	-	8.96	-1.71	-

Supplementary Table 2.4. Candidate genes for behavioral QTL. Names of candidate genes underlying behavioral QTL with known mutations between the two inbred strains. a. Maximum posterior probability of directional selection among 5kb windows within gene region (taken from Payseur and Jing (2020)). b. Number of mutations at sites conserved across placental mammals.

Gene	Chromosome	Traits with QTL	Max Probability of Selection[a]	Number of Mutations in Conserved Sites[b]	Number of Protein Coding Changes	Position in Protein Sequence	Amino Acid in Mainland	Amino Acid in Gough
Steap3	1	Distance Traveled Juvenile	0.025	6	1	465	N	S
Hnmt	2	Distance Traveled Juvenile, Distance Traveled Adult, Center Crosses	<0.01	9	3	12	Q	H
						20	R	Q
						164	A	D
Phf21a	2	Time in Center Juvenile, Distance Traveled Adult, Center Crosses	<0.01	9	1	113 (insertion)	-	Q
Plcb1	2	Fecal Boli Adult, Fecal Boli Total	<0.01	54	2	985	H	Q
						1180	G	S
Gria2	3	Time in Center	<0.01	5	0	–	–	–

		Juvenile, Fecal Boli Adult						
Frrs1l	4	Time in Center Adult, Distance Traveled Adult	<0.01	1	0	–	–	–
Mpdz	4	Time in Center Adult, Center Crosses	<0.01	27	8	219	L	V
						691	G	V
						801	H	R
						914	R	S
						971	T	I
						1318	F	L
						1338	V	M
						1433	I	N
Dab1	4	Center Crosses	0.99	142	1	366	F	Y
Eya3	4	Distance Traveled Adult, Center Crosses	<0.01	0	1	185	T	A
Mapk10	5	Fecal Boli Juvenile, Fecal Boli Adult, Fecal Boli Total	<0.01	5	0	–	–	–
Dao	5	Fecal Boli Adult, Time in Light Chamber	<0.01	6	2	157	K	N
						295	H	R

Cadps2	6	Time in Center Juvenile	0.93	3	1	28	V	G
Magel2	7	Center Crosses	<0.01	0	1	516	P	A
Ctbp2	7	Distance Traveled Adult	<0.01	1	0	–	–	–
Chrnb3	8	Time in Light Chamber, Center Crosses	<0.01	4	1	170	K	R
Gnao1	8	Time in Center Juvenile, Time in Center Adult, Distance Traveled Adult	<0.01	16	0	–	–	–
Grm2	9	Distance Traveled Adult, Time past Center	<0.01	5	0	–	–	–
Syn3	10	Light Chamber Entrances	0.9	5	0	–	–	–
Gabrg2	11	Time in Center Juvenile	<0.01	2	0	–	–	–
Gabra1	11	Time in Center Juvenile	<0.01	1	0	–	–	–

Gabrb2	11	Time in Center Juvenile	<0.01	4	0	–	–	–
Pcdh17	14	Time in Center Juvenile, Time in Center Adult, Distance Traveled Adult, Center Crosses	0.011	7	0	–	–	–
Lsamp	16	Distance Traveled Adult	0.584	7	0	–	–	–
Slit1	19	Distance Traveled Juvenile, Distance Traveled Adult	<0.01	13	1	1073	L	P

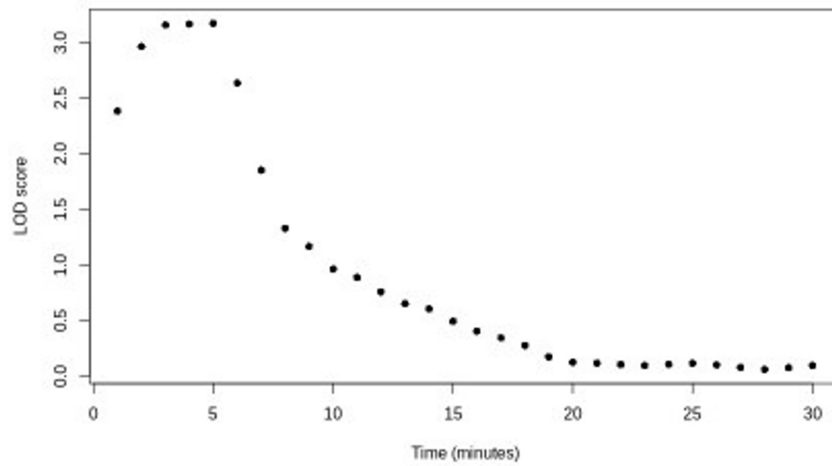
Supplementary Table 2.5. Genes annotated with “behavior” underneath each QTL (see “Candidate Gene Nomination” in Supplementary File 2.1). Supplementary Table 2.5 is available online at https://github.com/jeredastrat/gough_mouse_behavior/tree/main/Stratton_et_al_2023

Appendix C

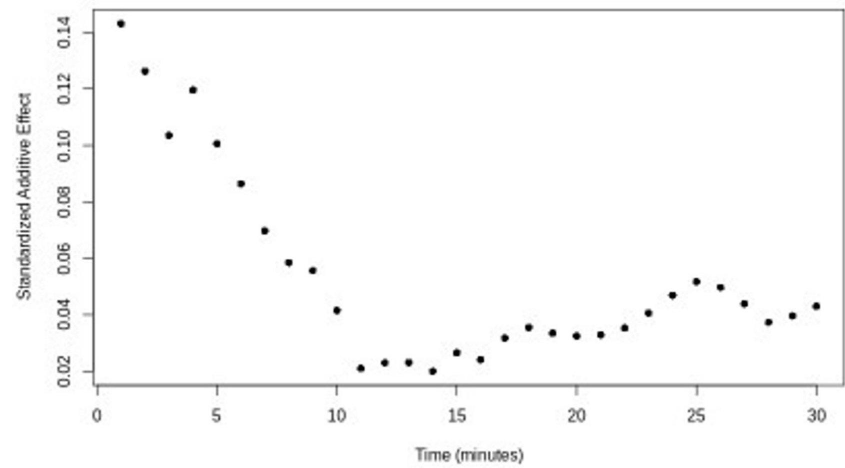
Supplementary Material for Chapter 3

Supplementary Figure 3.1. Temporal dynamics of iQTL on chromosome 14 for distance traveled in adult females. A. LOD score of iQTL calculated at each minute of the behavioral assay. B. Standardized additive effect (A/sigma) of iQTL estimated at each minute of the behavioral assay.

A



B



Supplementary Table 3.1. Genes associated with "abnormal social behavior" underneath iQTL.

Gene Symbol	Trait	Chromosome	Gene Name	MGI ID
Ambp	Week 9 Weight	4	alpha 1 microglobulin/bikunin precursor	MGI:88002
Brinp1	Week 9 Weight	4	bone morphogenic protein/retinoic acid inducible neural specific 1	MGI:1928478
Dmrta1	Week 9 Weight	4	doublesex and mab-3 related transcription factor like family A1	MGI:2653627
Plin2	Week 9 Weight	4	perilipin 2	MGI:87920
Aff4	Time in Light Chamber Males	11	AF4/FMR2 family, member 4	MGI:2136171
Dlg4	Time in Light Chamber Males	11	discs large MAGUK scaffold protein 4	MGI:1277959
Gria1	Time in Light Chamber Males	11	glutamate receptor, ionotropic, AMPA1 (alpha 1)	MGI:95808
Nlgn2	Time in Light Chamber Males	11	neuroligin 2	MGI:2681835
Pmp22	Time in Light Chamber Males	11	peripheral myelin protein 22	MGI:97631
Slc6a4	Time in Light Chamber Males	11	solute carrier family 6 (neurotransmitter transporter, serotonin), member 4	MGI:96285
Arsk	Time in Light Chamber	13	arylsulfatase K	MGI:1924291
Cmya5	Time in Light Chamber	13	cardiomyopathy associated 5	MGI:1923719
Gcnt4	Time in Light Chamber	13	glucosaminyl (N-acetyl) transferase 4, core 2 (beta-1,6-N-acetylglucosaminyltransferase)	MGI:2684919
Hexb	Time in Light Chamber	13	hexosaminidase B	MGI:96074
Ndufs4	Time in Light Chamber	13	NADH:ubiquinone oxidoreductase core subunit S4	MGI:1343135
Rnf180	Time in Light Chamber	13	ring finger protein 180	MGI:1919066
Whrn	Week 9 Weight	4	whirlin	MGI:2682003
Il5	Time in Light Chamber Males	11	interleukin 5	MGI:96557

Nek8	Time in Light Chamber Males	11	NIMA (never in mitosis gene a)-related expressed kinase 8	MGI:1890646
Srr	Time in Light Chamber Males	11	serine racemase	MGI:1351636
Ocln	Time in Light Chamber	13	occludin	MGI:106183
Slc6a3	Time in Light Chamber	13	solute carrier family 6 (neurotransmitter transporter, dopamine), member 3	MGI:94862

Supplementary Table 3.1 continued. Genes associated with "abnormal social behavior" underneath iQTL.

Gene Symbol	Start bp (GRCm39)	End bp (GRCm39)	QTL Peak Position	Distance from midpoint to peak (Mbp)	Number of missense variants	Number of conserved intergenic variants	Number of conserved genic variants	Number of conserved element changes	Interesting?
Ambp	63061512	63072409	95226892	32.15993	0	3	1	4	TRUE
Brinp1	68679751	68872634	95226892	26.4507	0	2	3	5	TRUE
Dmrta1	89576435	89583003	95226892	5.647173	0	15	0	15	TRUE
Plin2	86566623	86588297	95226892	8.649432	0	2	0	2	TRUE
Aff4	53241660	53312657	58425904	5.148746	0	2	2	4	TRUE
Dlg4	69908029	69938107	58425904	11.49716	0	0	2	2	TRUE
Gria1	56902342	57221070	58425904	1.364198	0	2	23	25	TRUE
Nlgn2	69713949	69728610	58425904	11.29538	0	0	1	1	TRUE
Pmp22	63019808	63050373	58425904	4.609187	0	6	3	9	TRUE
Slc6a4	76889429	76923166	58425904	18.48039	0	0	1	1	TRUE
Arsk	76208541	76246779	109659638	33.43198	0	0	1	1	TRUE
Cmya5	93177221	93281232	109659638	16.43041	30	2	23	25	TRUE
Gcnt4	97061197	97087414	109659638	12.58533	0	0	3	3	TRUE
Hexb	97312839	97334865	109659638	12.33579	0	2	0	2	TRUE
Ndufs4	114424331	114524630	109659638	4.814843	0	18	15	33	TRUE
Rnf180	105267075	105431406	109659638	4.310398	0	3	8	11	TRUE
Whrn	63333147	63414228	95226892	31.8532	0	0	0	0	FALSE
Il5	53611621	53615930	58425904	4.812129	0	0	0	0	FALSE
Nek8	78056932	78067501	58425904	19.63631	0	0	0	0	FALSE
Srr	74797185	74816774	58425904	16.38108	0	0	0	0	FALSE
Ocln	100633015	100689226	109659638	8.998518	0	0	0	0	FALSE
Slc6a3	73684866	73726791	109659638	35.95381	0	0	0	0	FALSE

Supplementary Table 3.2. Genes underneath iQTL on chromosome 14.

Gene Symbol	Gene Name	MGI ID	Start bp (GRCm39)	End bp (GRCm39)	QTL Peak Position	Distance from midpoint to peak (Mbp)
Abcc4	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	MGI:2443111	118720104	118943631	106437870	12.394
Cldn10	claudin 10	MGI:1913101	119025283	119111937	106437870	12.63074
Dct	dopachrome tautomerase	MGI:102563	118250202	118289656	106437870	11.83206
Dnajc3	DnaJ heat shock protein family (Hsp40) member C3	MGI:107373	119175388	119219109	106437870	12.75938
Dzip1	DAZ interacting protein 1	MGI:1914311	119112932	119162872	106437870	12.70003
Frg2f4	FSHD region gene 2 family member 4	MGI:3644083	106330668	106330916	106437870	0.107078
Gm9376	predicted gene 9376	MGI:3643023	118504570	118505191	106437870	12.06701
Gpc5	glypican 5	MGI:1194894	115329647	116762591	106437870	9.608249
Gpc6	glypican 6	MGI:1346322	117162727	118213956	106437870	11.25047
Gpr180	G protein-coupled receptor 180	MGI:1930949	118374570	118400673	106437870	11.94975
Hs6st3	heparan sulfate 6-O-sulfotransferase 3	MGI:1354960	119375753	120107227	106437870	13.30362
Ndfip2	Nedd4 family interacting protein 2	MGI:1923523	105496008	105546732	106437870	0.9165
Obi1	ORC ubiquitin ligase 1	MGI:1919736	104714972	104760081	106437870	1.700344
Pou4f1	POU domain, class 4, transcription factor 1	MGI:102525	104699112	104705435	106437870	1.735597
Rbm26	RNA binding motif protein 26	MGI:1921463	105344187	105414763	106437870	1.058395
Slitrk1	SLIT and NTRK-like family, member 1	MGI:2679446	109147420	109151671	106437870	2.711676
Slitrk5	SLIT and NTRK-like family, member 5	MGI:2679448	111912547	111920576	106437870	5.478692
Slitrk6	SLIT and NTRK-like family, member 6	MGI:2443198	110986012	110992581	106437870	4.551427
Sox21	SRY (sex determining region Y)-box 21	MGI:2654070	118470645	118474442	106437870	12.03467
Spry2	sprouty RTK signaling antagonist 2	MGI:1345138	106129381	106134253	106437870	0.306053

Tgds	TDP-glucose 4,6-dehydratase	MGI:1923605	118349323	118370167	106437870	11.92188
Trim52	tripartite motif-containing 52	MGI:3045276	106343632	106373293	106437870	0.079408
Uggt2	UDP-glucose glycoprotein glucosyltransferase 2	MGI:1913685	119222451	119336842	106437870	12.84178
A830021K08Rik	RIKEN cDNA A830021K08 gene	MGI:2443998	118943065	118945013	106437870	12.50617
Ednrb	endothelin receptor type B	MGI:102720	104052061	104081838	106437870	2.370921

Supplementary Table 3.2 continued. Genes underneath iQTL on chromosome 14.

Gene Symbol	Number of missense variants	Number of splice region variants	Number of conserved genic variants	Number of conserved intergenic variants	Number of conserved element changes	Interesting?
Abcc4	0	0	2	17	19	TRUE
Cldn10	0	0	0	3	3	TRUE
Dct	0	0	1	2	3	TRUE
Dnajc3	0	0	0	1	1	TRUE
Dzip1	0	0	0	3	3	TRUE
Frg2f4	0	0	0	1	1	TRUE
Gm9376	1	0	1	11	12	TRUE
Gpc5	0	0	33	2	35	TRUE
Gpc6	0	0	114	11	125	TRUE
Gpr180	0	0	0	7	7	TRUE
Hs6st3	0	0	21	1	22	TRUE
Ndfip2	0	0	8	0	8	TRUE
Obi1	0	0	1	35	36	TRUE
Pou4f1	0	0	6	35	41	TRUE
Rbm26	0	0	2	4	6	TRUE
Slitrk1	1	0	2	11	13	TRUE
Slitrk5	0	0	14	1	15	TRUE
Slitrk6	0	0	0	3	3	TRUE
Sox21	0	0	1	9	10	TRUE
Spry2	0	0	1	7	8	TRUE
Tgds	0	0	0	7	7	TRUE
Trim52	0	0	4	5	9	TRUE
Uggt2	0	0	1	1	2	TRUE
A830021K08Rik	0	0	0	0	0	FALSE
Ednrb	0	0	0	0	0	FALSE

Supplementary Table 3.3. Covariates used for each trait in iQTL mapping.

Trait	Sex	Parturition Number	Litter Size	Mother	Time of Day	Estrous	Temperature
Body Weight	X	X	X	X			
Distance Traveled Open Field Adult	X				X	X	
Time in Light Chamber			X				X

Supplementary Table 3.4. Direct QTL for body size.

Age	Chr	LOD score	Location (bp, GRCm39)	Lower bound position (bp, GRCm39)	Upper bound position (bp, GRCm39)	Additive effect (A)	SE(A)	Trait Standard Deviation (Sigma)	A/Sigma	SE (A/Sigma)
Week 4	1	1.171	23222314	22073787	25571200	0.586	0.081	2.029997	0.28867	0.039902
Week 4	6	6.167	24900730	3371546	42911794	0.409	0.078	2.029997	0.201478	0.038424
Week 4	7	6.67	49031692	40144117	70672202	0.407	0.085	2.029997	0.200493	0.041872
Week 4	8	4.507	90969836	49199979	126642669	0.37	0.083	2.029997	0.182266	0.040887
Week 4	10	9.785	121552668	116983686	126353795	0.515	0.078	2.029997	0.253695	0.038424
Week 4	11	5.189	9642197	3546803	33487377	0.377	0.083	2.029997	0.185715	0.040887
Week 4	13	5.298	71795947	53304991	102946983	-0.358	0.081	2.029997	-0.17635	0.039902
Week 4	16	7.086	6934401	3533018	12491603	0.426	0.083	2.029997	0.209853	0.040887
Week 5	1	13.97245	74018528	63354878	77902872	0.693	0.087	2.425268	0.285742	0.035872
Week 5	6	11.92057	24900730	18610928	31347790	0.628	0.086	2.425268	0.25894	0.03546
Week 5	7	6.37182	43562873	39903484	52160464	0.454	0.093	2.425268	0.187196	0.038346
Week 5	8	5.35205	69163522	64935727	126389747	0.404	0.09	2.425268	0.16658	0.037109
Week 5	10	8.53271	120191606	116983686	128086931	0.521	0.086	2.425268	0.214822	0.03546
Week 5	11	6.64331	16223226	3546803	21307452	0.441	0.091	2.425268	0.181836	0.037522
Week 5	12	7.39416	16544774	9512758	25566699	-0.547	0.097	2.425268	-0.22554	0.039996
Week 5	12	4.96814	93709513	82667118	102648717	0.406	0.094	2.425268	0.167404	0.038759
Week 5	13	5.6499	82597266	53304991	98673545	-0.402	0.088	2.425268	-0.16575	0.036285
Week 5	16	8.70219	6895378	3533018	9650072	0.533	0.091	2.425268	0.21977	0.037522
Week 6	1	15.23031	63978529	42984145	76976222	0.689	0.083	2.34274	0.2941	0.035429
Week 6	2	6.99928	133307401	74899601	160860003	-0.451	0.084	2.34274	-0.19251	0.035855
Week 6	6	11.73193	24900730	18610928	37652648	0.588	0.081	2.34274	0.250988	0.034575
Week 6	7	7.47728	45080867	39903484	52620167	0.492	0.088	2.34274	0.210011	0.037563
Week 6	8	5.26267	69408408	43790674	99391051	0.403	0.085	2.34274	0.172021	0.036282
Week 6	10	10.17747	120196040	116983686	126353795	0.541	0.081	2.34274	0.230926	0.034575
Week 6	11	6.99512	16223226	3546803	24424777	0.456	0.086	2.34274	0.194644	0.036709
Week 6	12	5.7782	16597066	3651849	25566699	-0.443	0.089	2.34274	-0.18909	0.03799

Week 6	12	5.34524	102502249	88956645	108361961	0.363	0.085	2.34274	0.154947	0.036282
Week 6	13	6.34062	82597266	58224670	107341960	-0.405	0.083	2.34274	-0.17287	0.035429
Week 6	16	6.14423	6987053	3533018	16242618	0.381	0.086	2.34274	0.16263	0.036709
Week 7	1	1.35	43900422	42333047	54690918	0.683	0.091	2.46172	0.277448	0.036966
Week 7	2	6.785	76080607	63780284	151894892	-0.51	0.095	2.46172	-0.20717	0.038591
Week 7	4	8	132623838	125219734	134582479	-0.623	0.111	2.46172	-0.25308	0.04509
Week 7	4	5.504	81559100	55597211	103938784	0.511	0.11	2.46172	0.207578	0.044684
Week 7	6	9.87	23447801	18610928	38982062	0.583	0.088	2.46172	0.236826	0.035747
Week 7	7	7.352	46282720	16755950	94580492	0.531	0.094	2.46172	0.215703	0.038185
Week 7	8	8.536	68700556	60820456	75886882	0.543	0.092	2.46172	0.220577	0.037372
Week 7	9	7.744	86197981	72960135	92273519	0.508	0.089	2.46172	0.20636	0.036154
Week 7	10	7.208	120196040	119388198	128086931	0.498	0.089	2.46172	0.202298	0.036154
Week 7	11	7.937	3546803	3546803	16948072	0.551	0.094	2.46172	0.223827	0.038185
Week 7	13	4.706	119002632	75050340	119392941	-0.377	0.091	2.46172	-0.15314	0.036966
Week 8	1	15.68	43900422	42333047	66189215	0.775	0.093	2.522976	0.307177	0.036861
Week 8	2	5.982	71328867	63626853	136265477	-0.484	0.097	2.522976	-0.19184	0.038447
Week 8	4	12.07	132636763	129468978	134582479	-0.755	0.11	2.522976	-0.29925	0.043599
Week 8	4	8.514	69035593	62955002	84852632	0.645	0.107	2.522976	0.25565	0.04241
Week 8	6	12.21	36989222	22215577	39487979	0.66	0.09	2.522976	0.261596	0.035672
Week 8	7	7.283	17384137	4144649	50180678	0.535	0.096	2.522976	0.212051	0.03805
Week 8	8	11.94	61610067	60820456	69662643	0.689	0.094	2.522976	0.27309	0.037258
Week 8	9	8.201	86197981	80324943	91950027	0.52	0.092	2.522976	0.206106	0.036465
Week 8	11	7.207	3546803	3546803	24424777	0.537	0.096	2.522976	0.212844	0.03805
Week 8	13	5.153	118734112	98557879	119392941	-0.426	0.093	2.522976	-0.16885	0.036861
Week 8	14	7.106	19830455	14605842	46985340	-0.542	0.097	2.522976	-0.21483	0.038447
Week 9	1	11.81581	43900422	42333047	64068981	0.726	0.101	2.652648	0.273689	0.038075
Week 9	2	10.39612	63780284	63605777	68684014	-0.848	0.125	2.652648	-0.31968	0.047123
Week 9	2	5.81551	22448622	12853852	33103150	0.582	0.119	2.652648	0.219403	0.044861
Week 9	4	10.99227	132636763	124313406	135577294	-0.79	0.119	2.652648	-0.29782	0.044861
Week 9	4	6.64759	69121789	59732465	84484955	0.622	0.117	2.652648	0.234483	0.044107
Week 9	6	10.37864	36989222	21424695	39019622	0.657	0.097	2.652648	0.247677	0.036567

Week 9	7	6.08754	19297716	3207519	50629276	0.532	0.104	2.652648	0.200554	0.039206
Week 9	8	11.32889	61941376	57464462	69015907	0.725	0.102	2.652648	0.273312	0.038452
Week 9	9	7.7715	86197981	72960135	92386066	0.546	0.099	2.652648	0.205832	0.037321
Week 9	11	7.29672	6480989	3546803	19451183	0.567	0.104	2.652648	0.213749	0.039206
Week 9	13	5.6845	119002632	111713773	119392941	-0.469	0.101	2.652648	-0.1768	0.038075
Week 9	14	6.45373	10357914	14605842	49135677	-0.558	0.106	2.652648	-0.21036	0.03996

Supplementary Table 3.4 continued. Direct QTL for body size.

Age	Chromosome	Dominance Effect (D)	SE(D)	D/A	SE(D/A)	Percent Variance Explained
Week 4	1	0.062	0.116	0.106	0.198	4.015
Week 4	6	0.007	0.115	0.018	0.28	2.071
Week 4	7	0.262	0.117	0.644	0.323	2.245
Week 4	8	0.025	0.116	0.067	0.314	1.505
Week 4	10	0.001	0.115	0.002	0.224	3.332
Week 4	11	-0.203	0.116	-0.538	0.327	1.737
Week 4	13	0.195	0.114	-0.546	0.347	1.774
Week 4	16	-0.244	0.115	-0.572	0.296	2.388
Week 5	1	0.025	0.125	0.036	0.181	4.003001
Week 5	6	0.077	0.126	0.123	0.201	3.38851
Week 5	7	0.225	0.128	0.496	0.303	1.773453
Week 5	8	0.253	0.127	0.627	0.34	1.483887
Week 5	10	-0.132	0.126	-0.253	0.244	2.394424
Week 5	11	-0.347	0.128	-0.787	0.324	1.850919
Week 5	12	0.143	0.128	-0.262	0.238	2.065983
Week 5	12	-0.256	0.129	-0.63	0.342	1.375446
Week 5	13	0.22	0.128	-0.546	0.346	1.568233
Week 5	16	-0.231	0.125	-0.434	0.249	2.443556
Week 6	1	0.118	0.119	0.172	0.174	4.162061
Week 6	2	-0.136	0.119	0.301	0.273	1.853697
Week 6	6	0.085	0.12	0.145	0.204	3.163486
Week 6	7	0.109	0.12	0.222	0.25	1.983882
Week 6	8	0.1	0.12	0.247	0.3	1.384639
Week 6	10	-0.107	0.119	-0.198	0.221	2.728133
Week 6	11	-0.246	0.122	-0.539	0.28	1.852565
Week 6	12	0.088	0.121	-0.197	0.274	1.523246
Week 6	12	-0.304	0.12	-0.837	0.372	1.406803

Week 6	13	0.206	0.121	-0.509	0.322	1.675072
Week 6	16	-0.308	0.119	-0.809	0.367	1.621984
Week 7	1	0.278	0.129	0.407	0.195	3.909
Week 7	2	0.141	0.129	-0.277	0.258	1.915
Week 7	4	-0.276	0.137	0.443	0.232	2.268
Week 7	4	0.226	0.138	0.442	0.284	1.545
Week 7	6	0.105	0.13	0.18	0.223	2.819
Week 7	7	-0.126	0.129	-0.238	0.245	2.079
Week 7	8	0.247	0.131	0.455	0.252	2.425
Week 7	9	0.138	0.13	0.272	0.26	2.194
Week 7	10	-0.091	0.129	-0.182	0.26	2.038
Week 7	11	-0.148	0.131	-0.268	0.239	2.25
Week 7	13	0.206	0.131	-0.546	0.382	1.318
Week 8	1	0.205	0.131	0.265	0.171	4.529
Week 8	2	0.174	0.132	-0.36	0.281	1.663
Week 8	4	-0.389	0.139	0.515	0.196	3.436
Week 8	4	0.166	0.138	0.258	0.218	2.391
Week 8	6	-0.097	0.132	-0.147	0.201	3.479
Week 8	7	0.089	0.132	0.166	0.249	2.035
Week 8	8	0.034	0.135	0.05	0.196	3.398
Week 8	9	0.254	0.133	0.488	0.27	2.3
Week 8	11	-0.148	0.133	-0.275	0.25	2.013
Week 8	13	0.12	0.133	-0.281	0.322	1.428
Week 8	14	0.021	0.133	-0.039	0.246	1.984
Week 9	1	0.149	0.143	0.206	0.198	3.53006
Week 9	2	0.135	0.152	-0.16	0.177	3.08833
Week 9	2	-0.19	0.15	-0.327	0.264	1.696341
Week 9	4	-0.366	0.15	0.463	0.2	3.273218
Week 9	4	0.125	0.15	0.2	0.243	1.945475
Week 9	6	-0.077	0.142	-0.118	0.217	3.082923
Week 9	7	-0.056	0.142	-0.105	0.268	1.777611

Week 9	8	0.039	0.146	0.054	0.201	3.378
Week 9	9	0.263	0.143	0.481	0.277	2.284595
Week 9	11	-0.25	0.143	-0.441	0.261	2.140972
Week 9	13	0.228	0.145	-0.486	0.331	1.657263
Week 9	14	0.107	0.144	-0.192	0.26	1.887287

Supplementary Table 3.5. Raw measurements and metadata for F2s used in this study. Supplementary Table 3.5 is available online at https://github.com/jeredastrat/gough_mouse_behavior/tree/main/Stratton_IGE