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The genetic basis of variation in sexual aggression: Evolution versus social plasticity

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Abstract

Male sexual aggression towards females is a form of sexual conflict that can result in increased fitness for males through forced copulations (FCs) or coercive matings at the cost of female lifetime fitness. We used male fruit flies (*Drosophila melanogaster*) as a model system to uncover the genomic contributions to variation in FC, both due to standing variation in a wild population, and due to plastic changes associated with variation in social experience. We used RNAseq to analyse whole-transcriptome differential expression (DE) in male head tissue associated with evolved changes in FC from lineages previously selected for high and low FC rate and in male flies with varying FC rates due to social experience. We identified hundreds of genes associated with evolved and plastic variation in FC, however only a small proportion (27 genes) showed consistent DE due to both modes of variation. We confirmed this trend of low concordance in gene expression effects across broader sets of genes significant in either the evolved or plastic analyses using multivariate approaches. The gene ontology terms neuropeptide hormone activity and serotonin receptor activity were significantly enriched in the set of significant genes. Of seven genes chosen for RNAi knockdown validation tests, knockdown of four genes showed the expected effect on FC behaviours. Taken together, our results provide important information about the apparently independent genetic architectures that underlie natural variation in sexual aggression due to evolution and plasticity.

KEYWORDS
artificial selection, *Drosophila melanogaster*, forced copulation, gene expression, plasticity, RNAseq, sexual aggression

1 | INTRODUCTION

There are many diverse strategies that males and females use to increase their fitness, some of which may not align with the ideal fitness outcomes for their sexual partners. This fitness misalignment generates sexual conflict, which has been a subject of thorough research by evolutionary biologists (Arnqvist & Rowe, 2005; Chapman, 2006; Fricke et al., 2010). Such sexual conflict can be relatively inconspicuous, for example on a molecular scale after copulation has occurred, where male seminal proteins can have a marked influence on female behaviour in favour of the male's fitness and at a cost to the female's (Chapman et al., 1995; Wigby & Chapman, 2005). On the other hand, sexual conflict can be obvious, as in the case of male sexual strategies that involve sexual aggression, such as forced copulation (FC)

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with females, which result in not only a potentially suboptimal mate that the female is unable to reject, but also physical harm that may reduce the female's lifetime fitness. FC is prevalent among animals including spiders, insects, reptiles, birds and mammals (Johns et al., 2009; McKinney et al., 1983; McKinney & Evarts, 1998; McLean et al., 2016; Muller & Wrangham, 2009; Olsson, 2017; Shine et al., 2003; Smuts & Smuts, 1993; Thornhill, 1980). Sexually aggressive behaviours may represent an alternative mating strategy employed by males that would otherwise be outmatched by other males vying for females, or rejected by females themselves. For example, male sailfin mollies (*Poecilia latipinna*) use either a courtship or sneaker strategy depending on their genotype or social environment, with the sneaker strategy employing forced insemination without female cooperation (Farr et al., 1986; Fraser et al., 2014).

Sexual aggression may be an important target of sexual selection, and understanding the genetic underpinnings that contribute to its variation in populations can give us a better picture of how these behaviours evolve, how variation in such behaviours can persist, and how this variation may be associated with environmental variation. Recently, fruit flies (*Drosophila melanogaster*) have been used as a model for understanding variation in sexual aggression. Fruit fly sexual aggression, in the form of male FC of recently eclosed teneral females, was first observed in wild populations in the field (Markow, 2000). Teneral females have a soft cuticle and unexpanded wings, and are unable to prevent forced intromission or escape from persistent males. FC of teneral females is beneficial for males since they are able to sire offspring, but is detrimental to females due to negative effects on survival and reproduction (Dukas & Jongsma, 2012; Seeley & Dukas, 2011). There is clear variation in male tendency to force copulate that can be attributed to both genetic and environmental variation. Assays of FC rate in isogenic lines of fruit flies have shown that its broad-sense heritability is about 0.16 (Baxter et al., 2019), and variation present in wild populations is sufficient for rapid divergence in FC rate via artificial selection (Dukas et al., 2020). FC rates have also been shown to vary in flies from the same genetic background that have been exposed to different social environments prior to exposure to teneral females. Males housed with no females forcibly mate at a higher rate than males who have been housed with virgin females prior to testing (Baxter & Dukas, 2017), and this effect of social experience on FC rate is of a similar magnitude to the response to artificial selection across 20 generations (Dukas et al., 2020).

Having access to a genetically tractable model system that shows both genotypic variation and variation due to social plasticity in sexual aggression gives us an excellent opportunity to investigate the similarity in the mechanisms underlying these two modes of variation. The similarity of the changes in gene expression due to genotypic variation and plasticity may have important ramifications for trait evolution. For example, shared mechanisms of plastic and genotypic effects on a trait may indicate the facilitation of adaptive evolution through genetic assimilation (Scheiner & Levis, 2021; Waddington, 1942). Evidence for the co-option of genetic mechanisms that underlie plasticity facilitating adaptive evolution has been observed in zooplankton (*Daphnia melanica*) adaptation to introduced predators (Scoville & Pfrender, 2010), in aggression in honey bees (*Apis mellifera*; Alaux et al., 2009), and in sailfin molly male reproductive tactics (Fraser et al., 2014). It is also possible, however, that plasticity may hinder adaptive evolution (Huey et al., 2003; Price et al., 2003; Robinson & Dukas, 1999). For example, the African savannah butterfly, *Bicyclus anynana*, expresses distinct phenotypes in the dry and wet seasons, which are associated with large differences in gene expression. This species, however, shows no intrapopulation genetic variation in plasticity, which limits evolutionary change in face of climate change (Oostra et al., 2018). Examining whether effects on sexual aggression gene expression due to plasticity and evolution are concordant or discordant could help us reveal the association between plasticity and evolution in the context of sexual aggression. Looking at the degree of overlap in genetic underpinnings of genotypic and plastic effects on a trait can also focus attention on key genes whose expression modification are necessary in both mechanisms of variation. For example, in fruit flies, just a single gene (*Cyp6a20*) was found to influence both evolutionary (Dierick & Greenspan, 2006) and plastic effects on male-male aggression (Wang et al., 2008), indicating that it may be particularly important in modification of aggressive behaviour over genes that may only influence one of those mechanisms of variation.

In the present study, we used fruit flies as a model system for genotypic and plastic variation in sexual aggression (specifically male FC rate) to ask several questions: (1) which genes show differential expression (DE) due to evolved differences in FC rate, socially plastic differences in FC rate, or both; (2) which ontological terms are overrepresented in these sets of differentially expressed genes; (3) to what degree are gene expression changes similar (in terms of direction and magnitude) due to evolution and plasticity; and (4) do male flies with knocked down expression for candidate genes identified as important for FC variation show the expected effects on FC rate? To answer these questions, we used lineages of evolved male flies artificially selected specifically for high and low FC rate (Dukas et al., 2020), and used an established protocol to generate male flies with high and low FC rate due to prior social experience (Baxter & Dukas, 2017). We then performed whole-transcriptome RNAseq on samples of head tissue from these males, followed by differential gene expression analysis between males with low and high FC rates. Finally, we performed tests to validate chosen candidate genes using RNA interference knockdown lines.

2 | MATERIALS AND METHODS

2.1 | Modification of FC rate due to artificial selection and plasticity

We previously generated lineages of flies diverged in male FC rate as a result of 20 generations of artificial selection (Figure 1a). Each generation, we allowed previously isolated, 3-day old males to pursue newly eclosed females for a maximum of 2 h. In each of three

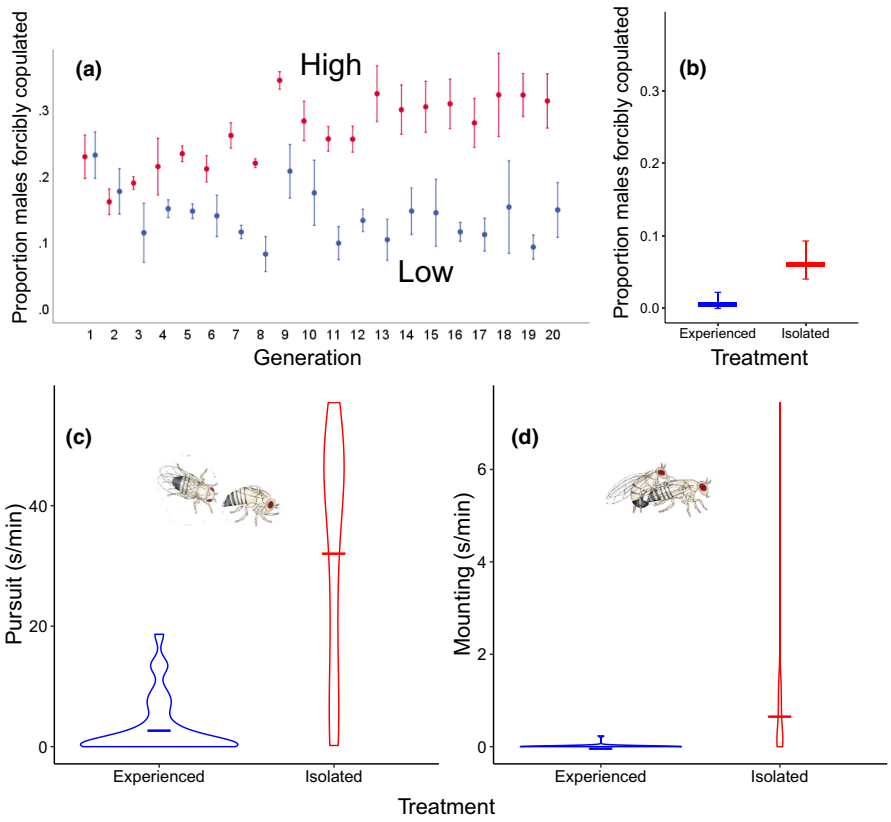

FIGURE 1 Evolved and plastic effects on FC. (a) Divergence in male FC rate (means ± SEMs) via artificial selection over 20 generations (based on data from: Dukas et al., 2020). Offspring of flies from generation 21 were snap frozen for RNA sequencing in this study. (b) Divergence in FC rate (proportion ± the standard error of the proportion p, $\sqrt{p(1-p)/n}$) of males from a wild-derived population as a result of prior social experience. This plasticity effect was also seen in behavioural determinants of FC. (c) Male pursuit of teneral females, and (d) male mounting of teneral females [Colour figure can be viewed at wileyonlinelibrary.com]

replicate lineages of the low FC males, we selected 48 males that did not mate with the teneral females and allowed them to mate with 48 unselected, mature females from their lineage to generate the next generation. In each of three replicate lineages of the high FC males, we selected 48 males that forcibly copulated with the teneral females and allowed them to mate with 48 unselected, mature females from their lineage to generate the next generation. Finally, in each of three control replicate lineages, we randomly selected 48 males and allowed them to mate with 48 mature females from their lineage to generate the next generation (Dukas et al., 2020). A similar effect on FC rate, in terms of direction and magnitude, can also be generated by varying the social environment males experience prior to exposure to teneral females (Baxter & Dukas, 2017). We first wished to verify this plastic effect on male FC rate as well as on potential determinants of FC success: pursuit of and mounting attempts on teneral females.

2.2 | Plastic effects of social experience on FC and its determinants

To test the effect of prior experience on FC rate and its determinants, we first sexed male flies under light CO_2 anaesthesia within 8 h of eclosion from a laboratory population of *D. melanogaster*. These flies were wild-caught in 2018, and from the same initial population that was used to generate the artificial selection lineages in Dukas et al. (2020), though not themselves subjected to artificial selection. These males were housed individually in vials with 5 ml standard food for 3 days (1 L standard food = 90 g sucrose, 75 g cornmeal, 10 g agar, 32 g yeast, and 2 g methyl paraben dissolved in 20 ml ethanol). We gave males in the experienced treatment a new 3-day old virgin female each day starting when the males were 1-day old without removing previous females, while males in the isolated treatment were left alone. We then tested the males when they were 4 days old, at which point the experienced males had been given three virgin females (figure 3a in Baxter & Dukas, 2017). Such sexually experienced males are still highly motivated to mate, as indicated by their moderate mating success with reluctant, recently mated females (figure 3d in Baxter & Dukas, 2017). Test arenas consisted of 35 mm Petri dishes coated with Surfasil (Thermo Fisher) on the walls and ceiling to keep flies on the bottom, with a circle of filter paper covering the bottom, and a thin food disc (5 mm diameter by 1.5 mm thick) with a small (1 mm) drop of yeast paste (1 part yeast:2 parts grapefruit juice) placed in the middle. Starting at 8:00 AM, we placed single isolated or experienced males with single teneral females from the same population into each test arena, and placed two arenas under each of five Logitech C920 webcams. We recorded arenas for 30 min, and then continued to manually scan for matings for 2 h after setting up. We tested 10 males (five from each experience treatment) per test session, and performed three test sessions per day over three consecutive days for a total $N = 90$ (45 per treatment). We recorded mating data for all 3 days, but video recorded only for the first 2 days due to a technology malfunction on day 3 (n for pursuit and mounting measurements = 30 per treatment).

Observers blind to treatment used BORIS behaviour observation software (version 7.9.8, Friard & Gamba, 2016) to record durations

that males spent pursuing and mounting teneral females. We defined pursuit as males following the teneral female with or without (usually with) singing, which was visible as wing vibration. We defined mounting as the male clearly arching his abdomen under and toward the teneral female, usually while grabbing onto the female, though this was not necessary. We analysed the mating data (i.e., whether the male forcibly copulated or not) with a generalized linear mixed model using GLMMTMB (version 1.0.2.1, Brooks et al., 2017) in R (version 4.1.0, R Core Team, 2021) with a binomial distribution, including treatment as a fixed effect, and random intercepts of test day, session, and arena. We changed the random intercept of session to a fixed effect to resolve model convergence issues. We similarly fit separate models for mounting duration and pursuit duration, which included video observer and day as fixed effects (as these variables had only two levels). The pursuit data had a large proportion of observations with 0 behaviour observed, and a right skew, so we fit these models with a Tweedie distribution, which fits these types of data well (Dunn & Smyth, 2005). We checked model assumptions with the simulateResiduals function from the DHARMA package (version 0.4.1, Hartig, 2020), tested the significance of the fixed effects in these models using the Anova function (CAR package, version 3.0-10, Fox & Weisberg, 2019) and report Wald χ^2 and associated *p*-values. We also calculated effect sizes using EMMEANS (Lenth, 2021).

2.3 | Fly collection for gene expression analysis

We collected male fly head tissue samples for RNA sequencing from both the FC artificial selection lineages, and from flies with varying social experience prior to exposure to teneral females. We collected males from the artificial selection lineages (those selected for high FC rate, low FC rate, and control) generated by Dukas et al. (2020) in generation 21, after 20 generations of artificial selection. We matched the morning timing and environmental conditions at collection to those used by Dukas et al. (2020), when flies would be tested for mating rate with teneral females, and included two conditions. In the teneral exposed condition, we gave 3-day-old males a single teneral female via mouth aspiration in a standard food vial with a foam plug lowered to 1.5 cm above the food to increase interactions. This condition closely matched the setup during the artificial selection study. We allowed the flies to interact for 10 min following first pursuit before snap freezing the males in liquid N_2. We wished to prevent the males from actually forcibly copulating, as mating would produce confounding effects on male gene expression (Ellis & Carney, 2010), and limiting the interaction duration to 10 min achieved this. In the teneral unexposed condition, 3-day-old males were not given a teneral female during the 10-min experience phase but were otherwise handled exactly as in the teneral exposed condition, including receiving a "sham" aspiration to simulate adding a teneral female with a mouth aspirator. Including a teneral unexposed condition allowed us to determine any DE effects among the selection treatments specifically in the presence of teneral females. Each sample prepared for extraction included 15 males of the same lineage and treatment combination collected during the same session (due to limited amounts of RNA in a single head). We collected three samples per lineage and treatment combination, at the level of maintenance vial (i.e., the artificial selection lineages were each maintained in 12 food vials, which were split into three groups of four vials for the purposes of collecting three vial-level replicates). In total, we collected 90 males from each of the nine lineages (three low FC, three control, and three high FC), 45 of which were teneral exposed and 45 teneral unexposed in three vial-level replicates. All samples of one replicate lineage from each selection treatment were collected per morning over three consecutive mornings. After snap freezing, we stored all 54 samples at −80°C until we removed heads and extracted RNA.

We then collected samples of male flies with diverged FC rate due to social plasticity in a similar manner. The laboratory population used, as well as rearing, timing, handling, and environmental conditions were matched to the experimental conditions described in the previous section, and we also included teneral exposed and teneral unexposed conditions. Sample collection was performed as previously described for the artificial selection lineages, with 90 males being collected for each experience treatment (isolated or socially experienced), 45 of each being teneral exposed, and 45 teneral unexposed. We collected two replicates for each treatment combination (1 per day over 2 consecutive mornings) from each of three sets of population rearing bottles, for a total of 24 samples. We wish to note the difference in the total number of samples collected for the artificial selection (54) and plasticity (24) experiments, although they are similar given the units of replication that are implied by the models used in the analysis (see the "Differential expression analysis" section below).

2.4 | RNA extraction and sequencing

We homogenized head tissues in 1.5 ml Eppendorf tubes using small metal beads and the NextAdvance Bullet Blender (NextAdvance). We extracted total RNA from heads using the Invitrogen MagMAX Total RNA Isolation Kit (Thermo Fisher) following kit specifications. This kit uses TRIzol (TRI reagent), followed by binding to magnetic beads to isolated RNA. We checked sample purity using a NanoDrop (ND 1000, Thermo Fisher) spectrophotometer and quantified concentrations with an Invitrogen Qubit RNA HS Assay Kit (Thermo Fisher) and DeNovix fluorometer (DeNovix). We then sent samples to the Génome Québec sequencing centre (Centre d'expertise et de services, Génome Québec) for library preparation and sequencing. Samples were then further assessed for quality and quantity of RNA using a Bioanalyser 2100 (Agilent). Libraries were prepared using NEB mRNA stranded Library preparation (using NEBNext dual multiplex oligos), and sequenced using a single lane Illumina NovaSeq 6000 S4 system (Illumina), using 100 bp paired-end sequencing technology. One sample from the plasticity set (socially experienced, teneral exposed) did not have usable RNA, and was therefore

not used in further analyses (although the two remaining replicates within this treatment combination were used). Samples had between 22.4 and 75.8 million reads, with an average of 36.6 million reads. We checked sample RNA quality, per-sequence GC content, duplication content, and adapter content using FASTQC (version 0.11.9, Andrews, 2019). The mean per-base Phred quality score of reads for all samples was >35. We trimmed adapters using TRIMMOMATIC (version 0.36, Bolger et al., 2014), with leading and trailing both set to "3", and with settings "MAXINFO:20:0.2". We then generated an index file based on the Flybase *D. melanogaster* transcriptome (version r6.34) for use with SALMON (version 1.1.0, Patro et al., 2017) to quasi-map RNAseq reads and generate count files of transcripts for each sample.

2.5 | Differential expression analysis

We imported count data into R using the TXIMPORT package (version 1.16.1, Soneson et al., 2015), which automatically summed counts to the gene level using the Flybase transcript-to-gene file (version 03/2020), such that counts for 13,758 genes were obtained. We computed offsets for the counts for use with downstream GLMs based on effective library sizes and transcript length, and we also filtered out lowly expressed genes (fewer than five counts). We then used two different DE analysis packages for use with our two types of data: NEBULA (version 1.1.7, He et al., 2021) which allows for the use of negative binomial generalized linear mixed effects modelling, which is necessary for the artificial selection data as these data include replicate lineages that need to be modelled as a random effect, and EDGER (version 3.34, Robinson et al., 2010) which allows for negative binomial generalized linear modelling for use with the plasticity data. The plasticity data were not analysed with NEBULA as flies were obtained from a single population, rather than from replicate lineages, and NEBULA requires a single random effect to be specified.

For the artificial selection count data, we fit a model of the form: count ~selection treatment (High FC, Low FC, Control) + teneral exposure (i.e., teneral exposed or teneral unexposed) + treatment:teneral exposure. NEBULA allows for a single random effect, which we included as replicate lineage. We originally included the effects of test day and vial-level replicate in the model; however, the resulting model coefficients were not estimable, and an extremely high condition number was produced. Therefore, we omitted day and vial-level replicate from the model, but did verify the estimates obtained from NEBULA by manually fitting models with GLMMTMB. We fit GLMMs using GLMMTMB for the top 200 genes obtained from the NEBULA main effect of selection treatment, which were of the form: count ~treatment + teneral exposure + treatment:teneral exposure, with random effects specified as: (1|Day) + (1 + teneral exposure|lineage/vial replicate). We included normalization factors calculated with the voom function (LIMMA package, version 3.48.0, Ritchie et al., 2015) as offsets. We ran a reduced model if the above specification produced inestimable coefficients: the same fixed effects, but with just the random intercept of lineage. The estimates from the NEBULA models and GLMMTMB models were highly correlated (r = .8 [95% CI: 0.74, 0.85]), so NEBULA estimates were used going forward in the analysis. We tested for DE genes first in the interaction between selection treatment and teneral exposure, however this revealed no significant DE genes (see Supplemental Methods and Results for further analysis of the lack of interaction). We then tested for DE genes in the high and low selection treatment contrast.

NEBULA does not have a built-in method for shrinking estimates to account for high biological variation, especially in genes with low mean expression. Therefore, we used the apeglm function from the APEGLM package (version 1.14.0, Zhu et al., 2019), which employs an empirical Bayes approach to shrink the estimates generated from NEBULA. We then obtained DE genes for the treatment contrast as above. We report both the results from the unshrunk and shrunken estimates for the artificial selection.

For the plasticity data set, we analysed the data using EDGER, which has a built-in empirical Bayes method to shrink gene-wise dispersions toward a global dispersion trend. We fit a model of the form: count ~treatment + teneral exposure + treatment:teneral exposure + day + vial replicate. As with the artificial selection, the initial analysis of the interaction term revealed no significant DE genes. Subsequent analyses are based on the significant DE genes from the main effect of treatment (isolated vs. socially experienced). As the number of DE genes obtained was still relatively low, we also included the significant DE genes based on the isolated versus experienced contrast within the group exposed to teneral females, which included some additional significant genes. We verified the accuracy of the EDGER estimates by comparing them to estimates obtained from identical models fit with limma-voom, and observed a high correlation between the estimates produced by these methods (r = .985, [95% CI: 0.980, 0.989], p < .0001). We also verified the accuracy of the estimates for the top hits obtained with EDGER by comparing them to estimates generated using GLMMTMB (as in the artificial selection analysis), which allowed us to specify vial as a random effect instead of as a fixed effect (as test day has only two levels in the plasticity analysis, it is not suitable to model as a random effect). The estimates obtained from the GLMMTMB models were highly correlated with those from EDGER (r = .97 [95% CI: 0.96, 0.98]), so estimates from EDGER were used going forward.

We wish to note that, while we have tailored the analyses of the artificial selection and plasticity DE to suit these individual data sets, the overall difference in methods used does present a caveat for comparisons between these analyses. To address this, we have included analyses focused as much as possible on the magnitudes of effects (see the section below on the "Comparison of directions and magnitudes of DE estimates due to artificial selection and plasticity").

2.6 | Gene ontology analysis

We performed gene ontology (GO) analysis on the sets of significant DE genes generated from the artificial selection and plasticity analyses, as well as the set of genes present in both (the overlap set). We

used the GO term list (version 05.2021) and the gene-GO association list (version 2.1) from Flybase, and the R package TOPGO (version 2.44.0; Alexa & Rahnenführer, 2016) to identify enriched GO terms in our sets of significant DE genes. We required GO terms to have at least five annotated genes to be included, and we used Fisher's exact test to test for significant enrichment. The *p*-values obtained here are not adjusted for multiple comparisons, which topGO does not perform, and a number of reasons are suggested by the package developers about why these corrections are not preferrable for GO analyses (see Alexa & Rahnenführer, 2016). For the GO results reported in Table 2, we also report FDR-adjusted *p*-values for reference. We also used topGO to graph the relationships of significant GO terms (Figures S2A, S2B and S2C).

2.7 | Comparison of directions and magnitudes of DE estimates due to artificial selection and plasticity

To get a better view of the overall degree of similarity in gene expression effects due to artificial selection and plasticity, we performed a vector correlation and magnitude analysis of: (1) the DE effects in the set of overlapping DE genes in each of the artificial selection and plasticity experiments, and (2) the DE effects in the broader set of genes significant in one experiment and the corresponding set in the other experiment (e.g., the DE effects in the significant genes in the plasticity Isolated-Experienced contrast, and the effects in the corresponding set in the artificial selection High-Low FC treatment contrast) regardless of significance in the latter experiment. This is analogous to the analysis performed in Zinna et al. (2018), and it allowed us to get a broader view of the similarity of the direction and magnitude of effects among the two mechanisms of FC behaviour change rather than simply using a more lenient false-positive rate, and specifically ask whether the DE effects due to plasticity also show correlated effects in those genes due to selection, and vice versa.

We calculated the vector correlations as $r_{VC} = \frac{|\boldsymbol{a} \cdot \boldsymbol{b}|}{\|\boldsymbol{a}\| \times \|\boldsymbol{b}\|}$ where $\boldsymbol{a}$ and $\boldsymbol{b}$ are vectors containing $\log_2$ fold changes (i.e., the estimates) obtained from relevant model contrasts (Zinna et al., 2018). For example, the estimates obtained for the set of significant DE genes in the plasticity comparison, and the estimates for the same set of genes in the artificial selection comparison. Vector correlation values close to 1 indicate a high concordance in the direction of the effects in the two comparisons for that set of genes, while values close to 0 indicate low concordance. We also calculated the value α for each of these vector comparisons as $\alpha = \frac{\|\boldsymbol{a}\|}{\|\boldsymbol{b}\|}$ which is the ratio of the magnitudes (L2 norms) of the vectors (Kuruvilla et al., 2002; Zinna et al., 2018), giving an estimate of the relative difference in the magnitude of DE effects between the two comparisons. Values close to 1 indicate a similar magnitude of DE effects for the two vectors, while values less than 1 indicate higher magnitudes in $\boldsymbol{b}$, and values greater than 1 indicate higher values in $\boldsymbol{a}$. In all of our analyses, the vector of estimates from the plasticity analysis was the numerator, so values greater than one correspond to larger magnitude of effects due to social experience.

We compared our observed r_{VC} and α values to empirical distributions of 10,000 such values generated by resampling estimates of the same number of genes from the entire set of genes (including the set of significant DE genes) as in Zinna et al. (2018). As described in that study, this approach is not a comparison to null expectations, and is instead a comparison of how extreme the observed values are to values obtained from vectors of the same length containing random estimates from the full set of genes. We considered observed values outside of the middle 95% of the distribution generated from this sampling to be extreme.

2.8 | Candidate gene choice and validation

We chose five genes from the set of overlapped DE genes significant in both the artificial selection and plasticity analyses as candidates for further validation of their effects on FC rate and pursuit of teneral females. These five genes were selected based on the following criteria: having the highest logFC estimates, concordant direction of effects in the artificial selection and plasticity analyses, and availability of mutants for candidate validation. This ruled out *lectin-28C* (RNAi lines not readily available) and *CG14025* (DE effects in opposite direction; Figure 4). We also selected one nonoverlapping gene from each of the artificial selection and plasticity significant DE gene lists for validation based on the same criteria (Figures S5 and S6 respectively).

TABLE 1 Genotypes used to generate crosses for candidate gene validation behavioural tests

Line	Genotype
RNAi-*CG14153*	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMJ22317}attP40
RNAi-*Drsl4*	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMC04568}attP40
RNAi-*GstZ1*	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS05870}attP2
RNAi-*Nepl18*	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMJ23000}attP40
RNAi-*verm*	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMC04570}attP40
RNAi-*Lsp2*	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMC04820}attP40
RNAi-*Nazo*	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS02717}attP40
elav-GAL-4	P{w[+mC]=GAL4-elav.L}2/CyO
TRiP control—attP2	y[1] v[1]; P{y[+t7.7]=CaryP}attP2
TRiP control—attP40	y[1] v[1]; P{y[+t7.7]=CaryP}attP40

We used RNAi knockdown lines crossed to a general nervous system GAL-4 to specifically knock down gene expression of our chosen candidate genes and observe the effects on FC rate and teneral pursuit. RNAi lines from the TRiP collection (Zirin et al., 2020) and the general nervous system GAL-4, elav-GAL4, were obtained from the Bloomington *Drosophila* Stock Centre (see Table 1 for genotypes). We generated three crosses for each candidate gene: TRiP-RNAi/elav-GAL-4, TRiP-RNAi/CyO (for simplicity, we refer to as TRiP-RNAi/+) and TRiP-control/elav-GAL-4 (+/elav-GAL-4). Conveniently, as the elav-GAL-4 line is maintained over a CyO balancer, crosses to this line generate the experimental cross TRiP-RNAi/elav-GAL-4 and the control TRiP-RNAi/+ cross in the same set of offspring. We do note that flies with CyO have curly wings, however we believe this has a negligible effect on FC rate as the ability to sing properly does not influence teneral female receptibility, since teneral females do not accept any matings regardless of male singing ability. Teneral females were reared from a laboratory population wild-caught in 2020.

We tested all three crosses for each gene concurrently, with testing for each gene spread over two consecutive days with an equal number of each cross tested per day. We sexed males under light CO_2 anesthesia within 8 h of eclosion and housed them individually in vials with 5 ml standard food for 4 days before testing. Starting at 8:00 AM we added a single teneral female to each male vial and lowered the vial plug to ~1 cm above the food to constrain the space and encourage interaction. We set up the vials for observation in vial racks in groups of 10, with all 10 being of the same genotype. We randomized the order racks were set up and counterbalanced the order between test days. An observer blind to genotype scanned all vials every 5 min for matings, and scanned a subset of vials every 10 min to record whether males were pursuing teneral females. Trials lasted until a FC occurred, or 2 h had elapsed. We aimed for 600 trials per gene (200 per cross), with a subset of ~240 of these (~80 per cross) also scanned for pursuit. Total sample sizes for each gene were as follows (with the subset scanned for pursuit in parentheses): *CG14153*—540 (230), *Drsl4*—580 (154), *GstZ1*—574 (243), *Nepl18*—479 (222), *verm*—577 (180), *Lsp2*—210 (143), *Nazo*—90 (87). Note that for the pursuit analyses, trials were excluded if there was a mating before the first pursuit scan, as in these cases there was no pursuit data. Sample sizes among genes varied due to teneral female availability, which was low in testing *Lsp2* and *Nazo* crosses. Sample sizes of crosses within each gene were nearly the same, ±<5%. Due to COVID-related restrictions at the time of this study, we were not able to verify the successful knockdown of these genes with qPCR. Zhang et al. (2021) demonstrated the need to include another analysis involving a set of genes that are expressed at similar levels but do not show gene expression differences between treatments. The lack of such analysis limits the interpretation of our results.

We analysed the mating data by fitting generalized linear mixed effects models for each gene using the GLMMTMB function and a binomial distribution, with the model specified as: mating (y/n) ~ Genotype + Day + (1|Vial rack). We also modelled the pursuit data using a binomial GLMM, and included whether a trial ended in a mating as an explanatory variable, as well as an observation-level variable (representing the time of the observation during the trial), and a trial ID as a random effect to account for repeated measures. These models took the form: pursuit (y/n) ~ Mated + Genotype + Day + Rack + Observation + (1 + Observation|Trial_ID). We checked model assumptions using the simulateResiduals function from the DHARMA package. We performed two contrasts: the first between the experimental genotype (RNAi/GAL-4) and the mean of the two control genotypes, and a second contrast between the two control genotypes. We computed the generalized inverse of these custom contrasts to get a contrast matrix, and hard coded this into the Genotype variable, to obtain *z* and *p*-values directly from the model summary after fitting.

3 | RESULTS

3.1 | Generation of flies with high and low FC success via artificial selection and environmental variation (plasticity)

We previously generated lineages of flies with significantly diverged FC rate using artificial selection (Dukas et al., 2020). As reported in that study, these males had on average a 0.15 FC rate in the low selection lineages versus a 0.3 FC rate in the high lineages (Figure 1a, generation 20, FC rate is over a 2-h period). In the present study, we were able to generate males with low and high FC rate by modifying the social environment experienced prior to exposure to teneral females. Isolated males had a higher, marginally nonsignificant FC rate compared to socially experienced males ($\chi^2_1 = 3.02$, $p = .08$, Cohen's $d = -1.92$ [95% CI: −4.13, 0.296], Figure 1b). The FC rate of the isolated males, however, was lower than the FC rate of 0.22 reported by Baxter and Dukas (2017), most probably owing to temporal variation. A closer analysis of male behaviours that typically precede FC revealed that isolated males also had significantly higher rates of pursuit of teneral females ($\chi^2_1 = 64.2$, $p < .001$, Cohen's $d = -0.194$ [95% CI: −0.256, −0.132], Figure 1c) and mounting attempts ($\chi^2_1 = 31.4$, $p < .001$, Cohen's $d = -0.73$ [95% CI: −0.994, 0.466], Figure 1d) compared to sexually experienced males.

3.2 | Gene expression in evolutionarily diverged and plastically different males

The contrast between low and high selection treatments revealed 903 significant DE genes using unshrunk estimates (Figure 2a,d), and 209 significant DE genes using shrunken (regularized) estimates (Figure 2b,e). Eighty-two genes were significant in both analyses of the unshrunk and shrunken estimates. The main effect of treatment in the plasticity analysis revealed 375 genes with significant DE between experienced and isolated males (Figure 2c,f). A small proportion of significant DE genes in either the artificial selection analysis (~0.02–0.05) or plasticity analysis (~0.03–0.05) were significant in both analyses (Figure 3a,b). In total, 27 genes were significantly DE

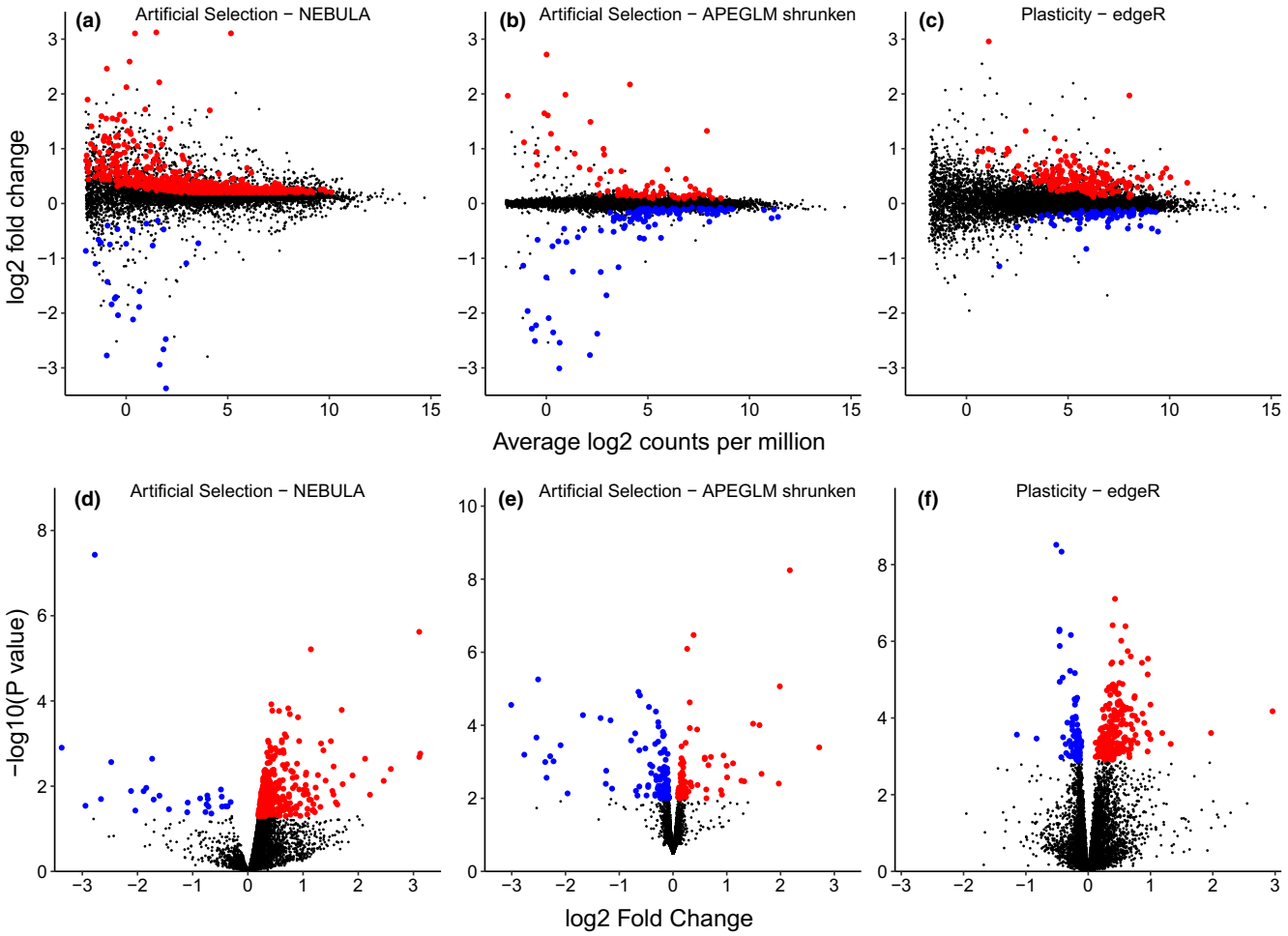

FIGURE 2 Differential expression of genes in flies with diverged FC tendency. The top row are MA plots showing the $\log_2$ fold changes as a function of mean $\log_2$ counts per million of (a) high/low FC lineages from the artificial selection, (b) high/low lineages using shrunken estimates, and (c) isolated/experienced plasticity treatments. The bottom row are volcano plots showing the $-\log_{10}$ *p*-values from the above contrasts as a function of log2 fold changes (as in corresponding plots above) for (d) artificial selection, (e) artificial selection using shrunken estimates, and (f) plasticity. Red dots indicate genes with significant upregulation in the high FC or isolated groups, and blue dots indicate genes with significant upregulation in the low FC or experienced groups [Colour figure can be viewed at wileyonlinelibrary.com]

in both artificial selection and plasticity (Figures 3a,b and 4; Figure S1A). Twenty-seven overlapped genes is not more extreme than the middle 95% of a distribution of 10,000 sets of overlapped genes each generated by randomly sampling without replacement 1030 genes for the artificial selection list and 375 genes for the plasticity list (median = 35 overlapped genes, 95% quantiles = [25, 47]). We found that 0 genes showed significant DE for the teneral present versus not present effect in the plasticity experiment, and 260 genes showed significant DE for that comparison in the artificial selection experiment using the unshrunk estimates (Figure S1B). Twelve were significant after shrinking estimates.

We performed GO analyses to identify ontological terms that are overrepresented in our samples of significant DE genes for the treatment effects (for GO terms with at least five total annotated genes). Fourteen terms were significantly overrepresented among significant DE genes in the artificial selection analysis, 35 terms were overrepresented in the plasticity analysis, and five terms were overrepresented in the 27 genes significant in both analyses (Table 2). Of particular note, in the overlapping genes set and plasticity set, neuropeptide hormone activity and general hormone activity were significantly enriched, and in the artificial selection set, serotonin receptor activity was enriched. GO graphs showing the relationship among significantly enriched terms are in Figures S2A, S2B and S2C.

3.3 | Comparison of direction and magnitude of gene expression between artificial selection and plasticity

Overall, we observed a low degree of similarity in the directions of DE among the 27 overlapping genes (Figure 5a, red bar), demonstrated by an observed vector correlation value that is not extreme relative to random subset of genes generated by vector correlations

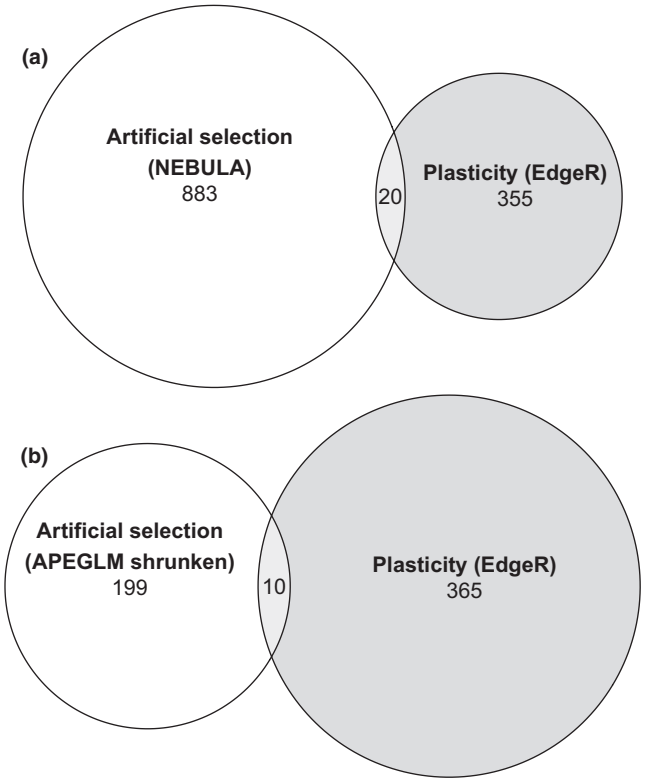

FIGURE 3 Genes with significant differential expression due to artificial selection and plasticity, and those significant in both. (a) Venn diagram of the significant artificial selection DE genes, significant plasticity genes, and overlap. (b) Venn diagram as in (a) except using shrunken estimates for determining significant artificial selection genes

of randomly sampled effects across all genes. Similarly, when looking at the entire set of significant DE artificial selection genes, or the set of significant DE plasticity genes, and the corresponding DE effects in the other experiment (i.e., plasticity and artificial selection respectively), the observed vector correlations are low and not outside of the middle 95% of the distribution generated from random sampling of all genes (Figure 5b black and grey bars; Figure S4A,B). These results were similar when using the set of significant artificial selection genes determined with shrunken estimates (Figure S3A), and when we controlled for a potential algorithmic effect due to using different analysis methods for the artificial selection and plasticity data (Figures S4A).

The magnitude of DE effects in the 27 overlap genes tended to be higher for plasticity compared to artificial selection, although this was again not more extreme than the middle 95% of the distribution generated from random sampling of all genes (Figure 5b, red bar). Overall, the set of significant artificial selection genes had higher magnitudes of DE effects in the artificial selection experiment, and the set of significant plasticity genes had higher magnitudes of DE effects in the plasticity experiment, compared to the corresponding magnitudes of DE effects in the plasticity and artificial selection experiments, respectively (Figure 5b black and grey bars; Figure S4C,D). This is unsurprising as there is little overlap of significant DE genes and low correlation of effects due to selection versus plasticity (Figures 4 and 5a). This magnitude difference was more extreme than that expected under our random sampling for the significant plasticity set, but not in the significant artificial selection set. When using shrunken artificial selection estimates (Figure S3B), and controlling for analysis method (Figure S3D), the results were generally similar. In this case, however, we also found a larger magnitude than expected from random sampling in the artificial selection effects for the significant artificial selection genes.

3.4 | Candidate gene choice and validation for genes contributing to variation in FC tendency due to AS and/or plasticity

The 27 genes showing significant DE due to both artificial selection and plasticity (Figures 3 and 4) were our starting point for choosing candidate genes for further analysis. We chose five genes from this list (Figure 4, red asterisks) based on a few criteria (see Methods). We also selected 1 nonoverlapping gene from each of the significant artificial selection and plasticity lists for validation (Figures S5 and S6 respectively; red asterisks). We wished to validate the effects of these seven genes (five overlap, one artificial selection, one plasticity) using RNA interference (RNAi) knockdown constructs crossed to a general brain targeted GAL4. All seven genes showed higher expression in the treatments with higher FC (High selection and isolated), so we expected the gene knockdown effect to manifest as a reduction in FC rate and pursuit of teneral females in RNAi/GAL4 crosses compared to RNAi and GAL4 controls.

Overall, four of the seven genes showed evidence of an effect in the predicted direction in at least one of: FC rate (Figure 6a,b) and pursuit of teneral females (Figure 6c,d). In the overlap set, knockdown of two of the five genes had the expected effect in FC rate (Figure 6a), and three of the five genes had the expected effect in pursuit (Figure 6c). In the experiment-specific set, only knockdown of the artificial selection gene (*Nazo*) produced an effect in the expected direction in both FC rate (Figure 6b) and pursuit (Figure 6d). *GstZ1*, *verm*, and *Nazo* showed a reduction of both FC rate and pursuit in the knockdown compared to controls (although the *Nazo* FC rate comparison was not significant due to a lower sample size). The *Nepl18* knockdown showed a reduction in pursuit but not FC rate. *CG14153* and *Drsl4* knockdowns showed the reverse pattern in FC rate (higher in knockdown vs. controls), but were not different from controls in pursuit.

4 | DISCUSSION

Our main findings were: (1) Variation in FC rate generated by evolution or plasticity (Figure 1) was associated with the significant DE of around 903 and 375 genes, respectively (Figures 2 and 3), (2) only 27 of these genes showed significant DE in both artificial selection and

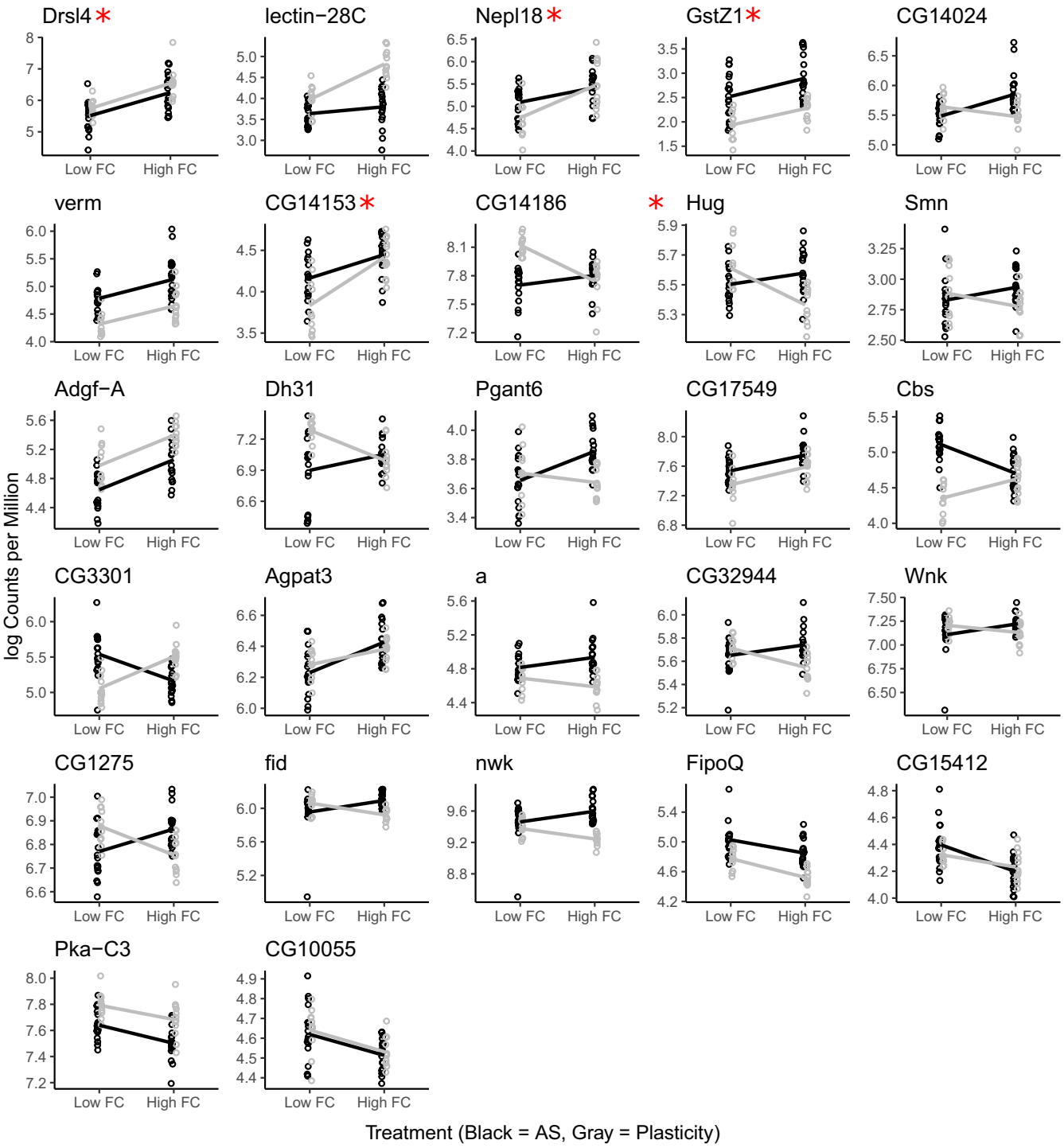

FIGURE 4 Genes that show significant differential expression due to both artificial selection and plasticity. The 27 genes that show significant differential expression in the artificial selection experiment (black lines/dots) and plasticity experiment (gray lines/dots). "Low FC" corresponds to both the low FC lineages (AS) and socially experienced (plasticity) treatments; "High FC" refers to the high FC lineages (AS) and socially isolated treatments (plasticity). The genes are ordered (left-right, top-bottom) by decreasing average log2 fold change values for the two experiments. Red asterisks indicate genes that we chose for follow-up candidate gene validation

plasticity (Figure 4), (3) significant DE genes in plasticity and these 27 overlapping genes showed enrichment in neuropeptide hormone and general hormone activity GO categories (Table 2), (4) the direction of gene expression effects in the set of significant DE genes for artificial selection were not correlated with the corresponding direction of effects in plasticity, or vice versa (Figure 5), and (5) four of seven candidate genes showed the predicted effects of gene knockdown on FC rate and pursuit of teneral females (Figure 6). We discuss each of these findings in turn, and suggest avenues for future research.

TABLE 2 Significantly enriched gene ontology (GO) terms in the sets of genes significant in both the artificial selection and plasticity analyses (27 overlapping genes), and each of the artificial selection and plasticity analyses separately

GO #	Gene ontology term	Annot.	Sig.	Exp.	*p*-value	*p* (FDR-adjusted)
Top GO groups from analysis of 27 overlapping genes						
GO:0005184	Neuropeptide hormone activity	30	2	0.08	.0025	1
GO:0005179	Hormone activity	39	2	0.1	.0043	1
GO:0016740	Transferase activity	1113	8	2.82	.0043	1
GO:0004674	Protein serine/threonine kinase activity	155	3	0.39	.0067	1
GO:0001664	G protein-coupled receptor binding	54	2	0.14	.0081	1
Top GO groups from analysis of AS top genes						
GO:0038187	Pattern recognition receptor activity	6	4	0.56	.00097	.332063
GO:0042805	Actinin binding	6	4	0.56	.00097	.332063
GO:0051393	Alpha-actinin binding	6	4	0.56	.00097	.332063
GO:0004930	G protein-coupled receptor activity	107	19	9.98	.00438	.553
GO:0038023	Signalling receptor activity	314	43	29.28	.00635	.553
GO:0060089	Molecular transducer activity	314	43	29.28	.00635	.553
GO:0004860	Protein kinase inhibitor activity	9	4	0.84	.00645	.553
GO:0004993	G protein-coupled serotonin receptor act…	9	4	0.84	.00645	.553
GO:0019210	Kinase inhibitor activity	9	4	0.84	.00645	.553
GO:0099589	Serotonin receptor activity	9	4	0.84	.00645	.553
GO:0004448	Isocitrate dehydrogenase activity	5	3	0.47	.007	.553
GO:0016886	Ligase activity, forming phosphoric ester…	5	3	0.47	.007	.553
GO:0051371	Muscle alpha-actinin binding	5	3	0.47	.007	.553
GO:0030247	Polysaccharide binding	10	4	0.93	.00997	.731371
Top GO groups from analysis of plasticity top genes						
GO:0016491	Oxidoreductase activity	551	46	20.48	1.40E−07	7.65E−05
GO:0003824	Catalytic activity	3316	168	123.26	1.50E−07	7.65E−05
GO:0005184	Neuropeptide hormone activity	30	9	1.12	8.70E−07	.000296
GO:0071855	Neuropeptide receptor binding	13	6	0.48	3.50E−06	.000893
GO:0005179	hormone activity	39	9	1.45	9.60E−06	.001649
GO:0030546	Signalling receptor activator activity	95	14	3.53	9.70E−06	.001649
GO:0030545	Receptor regulator activity	98	14	3.64	1.40E−05	.00204
GO:0017171	Serine hydrolase activity	218	22	8.1	1.90E−05	.002423
GO:0033764	Steroid dehydrogenase activity, acting o…	17	6	0.63	2.20E−05	.002448
GO:0001664	G protein-coupled receptor binding	54	10	2.01	2.40E−05	.002448
GO:0016229	Steroid dehydrogenase activity	19	6	0.71	4.50E−05	.004173
GO:0048018	Receptor ligand activity	90	12	3.35	.00011	.00935
GO:0004303	Oestradiol 17-beta-dehydrogenase activity	16	5	0.59	.00021	.016477
GO:0016614	Oxidoreductase activity, acting on CH-OH…	113	13	4.2	.00027	.019671
GO:0030297	Transmembrane receptor protein tyrosine…	5	3	0.19	.00048	.03264
GO:0016878	Acid-thiol ligase activity	21	5	0.78	.00086	.054
GO:0016616	Oxidoreductase activity, acting on the C…	97	11	3.61	.0009	.054
GO:0008236	Serine-type peptidase activity	165	15	6.13	.0012	.068
GO:0004252	Serine-type endopeptidase activity	151	14	5.61	.00144	.077305
GO:0030296	Protein tyrosine kinase activator activi…	7	3	0.26	.00159	.079171
GO:0008374	O-acyltransferase activity	24	5	0.89	.00163	.079171
GO:0030295	Protein kinase activator activity	25	5	0.93	.00198	.0918

(Continues)

TABLE 2 (Continued)

GO #	Gene ontology term	Annot.	Sig.	Exp.	*p*-value	*p* (FDR-adjusted)
GO:0019209	Kinase activator activity	26	5	0.97	.00238	.1054
GO:0016411	Acylglycerol O-acyltransferase activity	8	3	0.3	.00248	.1054
GO:0005102	Signalling receptor binding	255	19	9.48	.00301	.122808
GO:0016289	CoA hydrolase activity	9	3	0.33	.00362	.138064
GO:0015645	Fatty acid ligase activity	18	4	0.67	.00379	.138064
GO:0016405	CoA-ligase activity	18	4	0.67	.00379	.138064
GO:0004175	Endopeptidase activity	264	19	9.81	.0044	.154759
GO:0016877	Ligase activity, forming carbon-sulphur b...	30	5	1.12	.00456	.15504
GO:0016903	Oxidoreductase activity, acting on the a...	43	6	1.6	.0048	.157935
GO:0016620	Oxidoreductase activity, acting on the a...	31	5	1.15	.00527	.167981
GO:0050660	Flavin adenine dinucleotide binding	62	7	2.3	.00781	.2414
GO:0005342	Organic acid transmembrane transporter a...	80	8	2.97	.00946	.275691
GO:0046943	Carboxylic acid transmembrane transporte...	80	8	2.97	.00946	.275691

Note: Results show the total number of annotated genes in each category (Annot.), number of genes from our significant gene sets in each category (Sig.), number of genes expected to be significant in each category under a null hypothesis of no enrichment (Exp.), and associated *p*-values for the Fisher's exact tests (*p*). While *p*-values unadjusted for multiple comparisons are preferrable for GO analyses (Alexa & Rahnenführer, 2016), FDR-adjusted *p*-values are also provided for reference.

We identified a relatively large number of significant DE genes associated with diverged FC rate (Figure 2). This is in agreement of the growing body of literature showing that behavioural variation is associated with the DE of a large number of genes (Dierick & Greenspan, 2006; Gammie et al., 2007; Immonen et al., 2017; Shultzaberger et al., 2019), rather than a few genes with large effects. The vast majority of our observed differential gene expression effects appeared to be due to either the main effects of selection treatment or prior exposure to females, rather than the interaction of these effects with exposure to teneral females during the trial, or the main effect of exposure to teneral females. Of these genes, however, only 2%–5%, or 27 total genes, showed significant DE due to both artificial selection and plasticity (Figures 3 and 4). It is worth reiterating that, in the artificial selection, male experience was always isolation prior to testing, while in the plasticity, half of the males had no exposure while the other half had experience with females. One may argue that the female deprivation versus exposure triggers another set of genes than those involved in the tendency to engage in FC in female-deprived males. We know, however, that males with prior mating experience are not in a refractory period and are still motivated to mate, even with less willing females, as they mate at relatively high rates with recently mated females (Baxter & Dukas, 2017). Two previous studies have examined changes in gene expression in males either after courting mature females for 20 min (Ellis & Carney, 2011), or 2 h after mating (Ellis & Carney, 2010). The male courtship study is similar to our comparison between males exposed to teneral females versus unexposed males. The male mating study is similar to our plasticity treatments, where males were either allowed to mate for 3 days or kept isolated. We thus made two relevant comparisons between the list of differentially expressed genes reported by Ellis and Carney (2010, 2011) and those found in our study. Five genes that showed upregulation after males courted mature females (Ellis & Carney, 2011) were also upregulated after males pursued teneral females (Table S1). In contrast, all four differentially expressed genes found after mating in both Ellis and Carney (2010) and our study showed distinct responses (Table S1), suggesting that different patterns of gene expression underlie short and long term effects of mating on males.

Overall, the lack of overlap in DE due to both artificial selection and plasticity is in contrast to a few other findings comparing gene expression effects of trait variation due to evolution and plasticity. These studies have documented a high degree of overlap between evolved and plastic effects on mating strategies in sailfin mollies (*Poecilia latipinna*; Fraser et al., 2014), and on temperature effects in graylings (*Thymallus thymallus*; Mäkinen et al., 2016). However, in one other study of the evolved and plastic effects on gene expression in a fruit fly behaviour (male-male aggression), only a single gene was found to be significantly DE in both (Wang et al., 2008). This result remains ambiguous, however, as two other studies of male-male aggression did not identify this gene in an artificial selection experiment (Edwards et al., 2006), and in a plasticity experiment (Agrawal et al., 2020). The scarcity of data precludes generalizations at this point.

In the set of 27 genes significant in both evolved and plastic variation in FC rate, and in the set of significant plasticity genes, our GO analyses revealed that a few ontological categories related to neuropeptide hormone activity, and neuropeptide receptor binding, were significantly overrepresented (Table 2). Neuropeptides are a highly diverse set of chemical messengers that are well known to be involved in the regulation of many neural circuits involved in behaviour (Jékely et al., 2018; Nässel & Larhammar, 2013), and specifically in insect behaviours such as mating behaviour and feeding

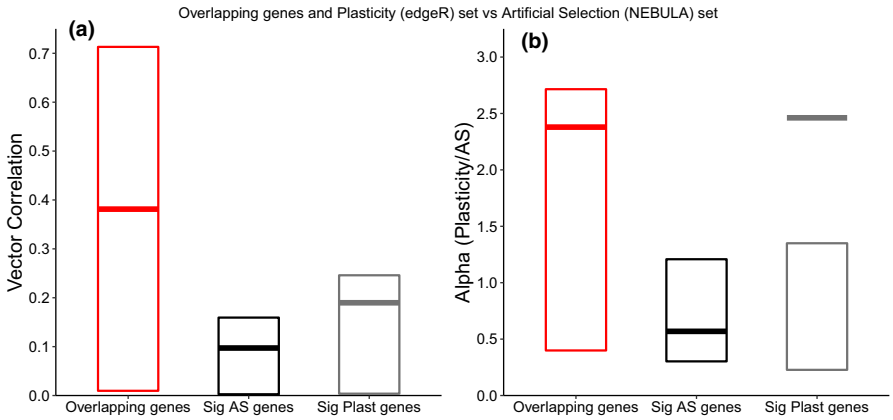

FIGURE 5 Similarity in direction and magnitude of DE estimates between artificial selection and plasticity. (a) The observed vector correlation values (thick horizontal lines) between vectors of estimates (log2 fold changes) obtained from the artificial selection and plasticity analyses for three sets of genes: the 27 genes significant in both (red), all the genes significant in artificial selection analysis and the corresponding estimates for those genes in the plasticity (black), and all the genes significant in the plasticity analysis and the corresponding estimates for those genes in the artificial selection (grey). Values close to 1 indicate a high concordance in the direction of the effects in the two comparisons for that set of genes, while values close to 0 indicate low concordance. (b) The observed ratio of vector magnitudes (plasticity/artificial selection), or alphas, for the same vector comparisons. Values close to 1 indicate a similar magnitude of DE effects for the two vectors, while values less than 1 indicate higher magnitudes in the artificial selection set, and values greater than 1 indicate higher values in the plasticity set. Rectangles represent the 95% least extreme estimates of the distribution generated from empirical resampling of estimates from all genes [Colour figure can be viewed at wileyonlinelibrary.com]

(Nässel, 2014; Schoofs et al., 2017; Taghert & Nitabach, 2012). They have also been shown to be important regulators of sexual behaviour in mammals. Neuropeptide manipulations in female prairie voles (*Microtus ochrohaster*) early in life are associated with changes in later life sexual behaviour, and neural responses to social stimuli (Cushing et al., 2005; Kramer et al., 2006). Many neuropeptide signalling systems are highly conserved and shared across widely diverse taxa (Elphick et al., 2018). It is possible that neuropeptide hormone regulation in response to prior social environments facilitates a plastic shift in mating behaviours, including FC tendency, and that this plastic mechanism may have been partly co-opted in producing genotypic variation via evolution. Knockdown of the fruit fly neuropeptide gene *Dsk* has been shown to increase social isolation-induced aggression (Agrawal et al., 2020), and while this gene was not specifically a significant hit in any of our experiments, the overrepresentation of such neuropeptide genes in our gene sets agrees with the overall body of knowledge that such neuropeptide systems are involved in regulating aggressive social behaviours. In addition, serotonin receptor activity was significantly enriched in the set of genes with significant evolved DE, indicating that the serotonergic system, or upstream regulators of it, may have been a target of the FC artificial selection regime. The serotonergic system is involved in fruit fly aggression (Alekseyenko et al., 2014, 2019; Dierick & Greenspan, 2007) as well as a wide variety of other behaviours, including feeding (Tierney, 2020) and sexual behaviour (Becnel et al., 2011), and may also be involved in the regulation of a stress-induced depression-like state (Ries et al., 2017). In addition, its involvement in sexual behaviour has been shown to be diverged in males and female rodents (Angoa-Pérez & Kuhn, 2015).

We further investigated the overall degree of similarity in gene expression effects between evolved and plastic changes in FC to see if there existed a broader pattern of concordant changes in expression that was not captured simply by looking at the overlapping significant DE genes. We found that the small degree of overlap between evolved and plastic effects extended to the broader sets of genes significant in either the artificial selection or plasticity analyses, as genes that were significant in one analysis did not tend to show a correlated (even if not significantly DE) effect in the other (Figure 5). A few studies reported a positive correlation between plastic and evolved effects on gene expression (Alaux et al., 2009; Fraser et al., 2014; Radersma et al., 2020; Scoville & Pfrender, 2010). In contrast to these studies, our results point to the relative independence of the mechanisms underlying variation in FC tendency for genotypic variation, and plastic variation. Future studies could look more directly at the behavioural differences in evolved versus plastic effects on FC to disentangle these potentially different mechanisms. Of interest in our study is despite the effect of social isolation on FC being about a third the magnitude of the effects of 20 generations of artificial selection, the magnitude of gene expressions are generally higher in the former, though not representing an extreme outcome relative to random subsets of genes (Figure 5b).

We then focused our attention on the 27 overlapping genes that may be part of a shared mechanism of FC regulation that contributes to both evolved and plastic differences. Of 5 genes chosen for follow-up tests with RNAi knockdown crosses, we were able to validate the effects of *GstZ1*, *Nepl18* and *verm* on FC rate and/or pursuit of teneral females using fly crosses containing gene specific knockdowns (Figure 6a,c). We were also able to validate the effect of *Nazo*, which had one of the largest DE magnitudes in the list of significant

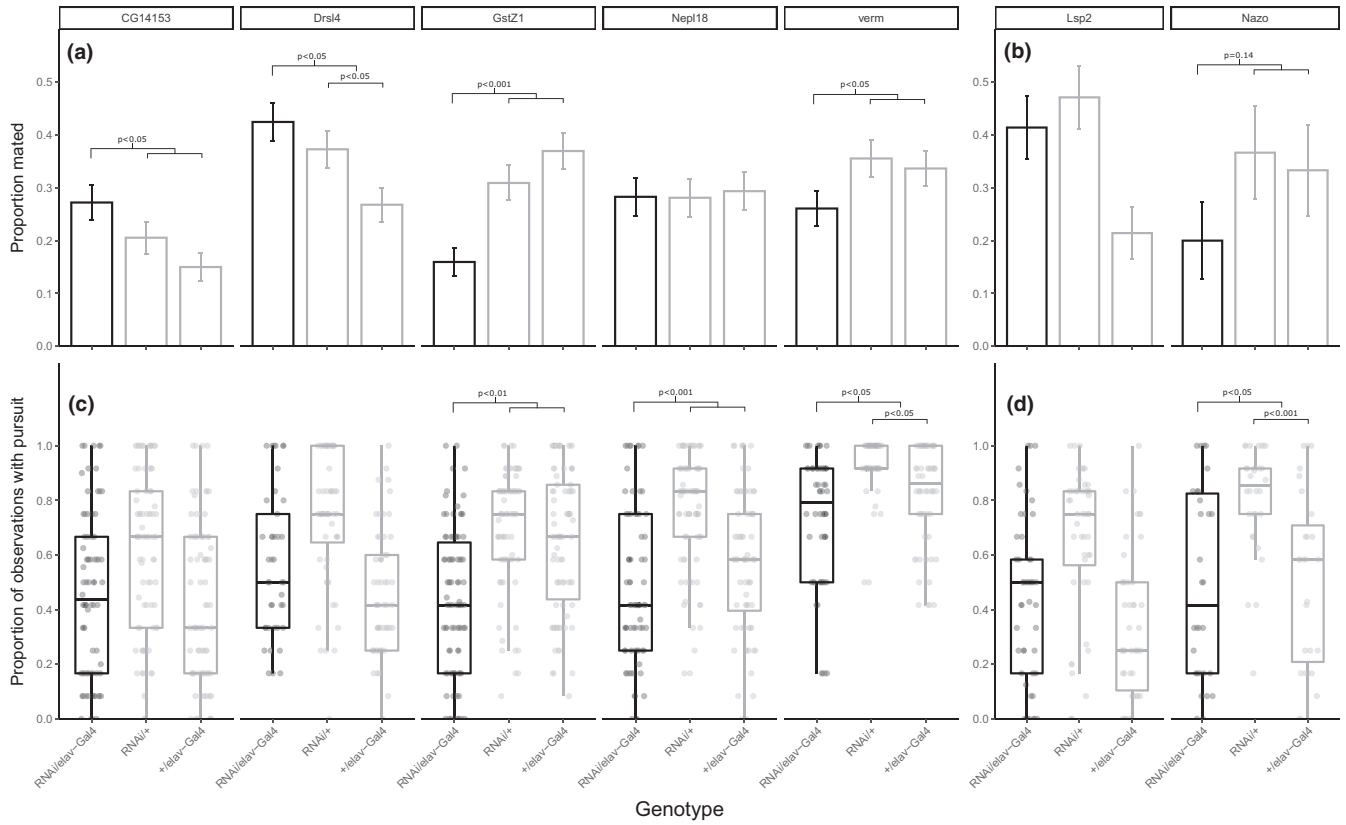

FIGURE 6 Functional validation of candidate genes that may contribute to variation of FC rate due to artificial selection and plasticity. Males from knockdown crosses (black) and two control crosses (grey) were measured for rate of FC (top row, proportion ± the standard error of the proportion p, $\sqrt{p(1-p)/n}$) and proportion of observations with pursuit of teneral females (bottom row). Seven genes were tested for effects on these behaviours in knockdown crosses: five that showed significant DE due to both artificial selection and plasticity (a, c), and two that were significant in one of those experiments (b, d; plasticity gene = Lsp2, artificial selection gene = Nazo). Significant contrasts between the knockdown cross and the mean of the two controls is shown above each plot. A significant contrast between the two controls is also shown if the knockdown-control contrast is significant

DE genes in the artificially selected flies list (Figure 6b,d). *GstZ1*, orthologous to human GSTZ1, is involved in enabling glutathione transferase activity (Saisawang et al., 2012), with no obvious direct link to behaviour. *Nepl18* is orthologous to mammalian Neprilysin, an endopeptidase which degrades amyloid beta, the buildup of which is a hallmark of Alzheimer's disease. Neprilysin-deficient mice have been shown to have neuronal degradation and weakened learning ability (Madani et al., 2006). The potential memory-related effects of *Nepl18* in fruit fly pursuit of teneral females is intriguing, though unclear, and requires further investigation. *verm*, also known as *hlm*, is expressed in the trachea and in photoreceptors, and the knockdown of this gene during fly development results in reduced visual acuity and poor optomotor response (McKay et al., 2008). The visual system has been shown to be important for successful male courtship (Markow, 1987), and it is possible that variation in visual acuity may be an important distinction between males that do and do not forcibly mate. Finally, *Nazo*, which was significantly DE in just the artificial selection experiment, is involved in neuromuscular processes and has been shown to result in reduced climbing ability in flies with knocked-down expression (Iuso et al., 2014). The DE observed among selection treatments for *Nazo* may therefore indicate the selection for alleles associated with physical ability to overcome teneral female resistance in the high FC males. Physical traits such as size and ornamentation have been shown to affect male ability to sexually coerce females (Crean & Gilburn, 1998; Perry & Rowe, 2012); however, no effect of selection was observed on the physical traits of sex comb number or body size in our artificially selected fruit fly lineages (Dukas et al., 2020). We have not, however, quantified more subtle physical characteristics such as strength. We do note that, since we expected the effects of the gene knockdowns to result in lower FC and pursuit rates compared to controls, it is possible that our positive results could be the consequence of an overall reduction in fly rigor, and so further tests on these flies' mating and courting rates with mature virgin females is required to rule out this explanation of the results.

Overall, we report here that the evolution of male FC success via artificial selection, and the effects on FC success from the social environment, are each associated with the DE of hundreds of genes. However, the degree of overlap in DE between evolved and plastic differences is minimal and only includes a small subset of potentially key genes. Further investigation into the functions and mechanisms of these overlapping genes, as well as genes that only

show DE in either evolution or plasticity, will be important in our understanding of the genetic architecture that is necessary for both evolved and plastic changes in FC success, and in our understanding of the genetic architecture that differentiates these two types of variation.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

Janice L. Yan, Carling M. Baxter and Reuven Dukas designed and ran the artificial selection experiment, Andrew M. Scott, Janice L. Yan, Ian Dworkin, and Reuven Dukas collected RNA samples from the artificial selection lineages, Andrew M. Scott, Ian Dworkin, and Reuven Dukas designed the other experiments, Andrew M. Scott ran the other experiments, Andrew M. Scott and Ian Dworkin analysed the data, Andrew M. Scott, Ian Dworkin, and Reuven Dukas wrote the first draft, and all authors contributed to the revisions.

OPEN RESEARCH BADGES

This article has been awarded Open Data Badges. All materials and data are publicly accessible via the Open Science Framework at https://doi.org/10.5061/dryad.zs7h44jbr and https://doi.org/10.5281/zenodo.6110067.

DATA AVAILABILITY STATEMENT

The raw sequence reads have been archived at NCBI BioProject under accession number PRJNA816348. All other data, including gene-level counts and data from behavioural experiments are archived at Dryad (doi: https://doi.org/10.5061/dryad.zs7h44jbr). The R and Bash scripts used for data analysis have been archived at Zenodo (doi: https://doi.org/10.5281/zenodo.6110067).

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