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Physiological and genetic correlates of boldness: characterising the mechanisms of behavioural variation in rainbow trout, *Oncorhynchus mykiss*

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Abstract

Bold, risk-taking animals have previously been putatively linked with a proactive stress coping style whereas it is suggested shyer, risk-averse animals exhibit a reactive coping style. The aim of this study was to investigate whether differences in the expression of bold-type behaviour were evident within and between two lines of rainbow trout, *Oncorhynchus mykiss*, selectively bred for a low (LR) or high (HR) endocrine response to stress, and to link boldness and stress responsiveness with the expression of related candidate genes. Boldness was determined in individual fish over two trials by measuring the latency to approach a novel object. Differences in plasma cortisol concentrations and the expression of eight novel candidate genes previously identified as being linked with divergent behaviours or stress were determined. Bold and shy individuals, approaching the object within 180 s or not approaching within 300 s respectively, were evident within each line, and this was linked with activity levels in the HR line. Post-stress plasma cortisol concentrations were significantly greater in the HR line compared with the LR line, and six of the eight tested genes were upregulated in the brains of LR fish compared with HR fish. However, no direct relationship between boldness and either stress responsiveness or gene expression was found, although clear differences in stress physiology and, for the first time, gene expression could be identified between the lines. This lack of correlation between physiological and molecular responses and behavioural variation within both lines highlights the complexity of the behavioural-physiological complex.

Keywords: boldness; cortisol; HPI axis; novel object; *Oncorhynchus mykiss*; qRT-PCR; stress coping styles.

Introduction

Behavioural polymorphisms are a common feature of natural populations (Sih et al., 2004). In some cases intraspecific variation in behaviour may be inherently necessary due to environmental changes, often corresponding with ontogenetic shifts (Slater, 1981). However, for many complex behaviours the full adaptive significance of such variation is not fully understood. Despite this, recent studies have highlighted the underlying role of physiological and genetic factors in driving divergent behaviour, particularly differences in animal personality (Bell, 2007; Koolhaas et al., 1999; Korsten et al. 2010; Øverli et al., 2005). One fundamental personality trait is boldness. An individual's boldness is defined by its response to a novel challenge, with these responses regarded as an indicator of the amount of risk an animal is prepared to take in new circumstances (Koolhaas et al., 1999; Sih et al., 2004; Sneddon, 2003, van Oers et al., 2005). As such, boldness can directly influence an organism's fitness, with costs or benefits dependent upon the environmental context (Brown et al., 2007).

Boldness is not a discrete trait, but rather represents a continuous range of behavioural profiles from bold to shy (Cockrem, 2007). This bold/shy continuum describes a suite of correlated behaviours which are often considered consistent between contexts. In general, shy animals are more reclusive or unresponsive when faced with an unfamiliar situation, whilst bold organisms will act normally or even actively investigate novel environments or objects more readily under the same conditions (Beausoleil et al., 2008; Carere and van Oers, 2004; Frost et al., 2007; Verbeek et al., 1994; Wilson et al., 1993; Yoshida et al., 2005). Bold animals are also relatively more aggressive, spend more time in the open, recover more quickly (e.g. from fear stimulation) and are able to learn more quickly than shy animals

(Carere et al., 2005; Magnhagen, 2007; Sneddon, 2003; van Oers et al., 2005; Verbeek et al., 1996).

Behavioural profiles within a species have also been linked with the physiological response to a stressor, collectively comprising the individual's 'coping style' (Koolhaas et al., 1999). Stressors are defined as challenges to an individual's homeostasis that result in a stress response: behavioural and neuroendocrine reactions that address the negative effects of that challenge (Wendelaar Bonga, 1997). Intraspecific differences in stress responsiveness reflect variation in the control of hormone release within the neuroendocrine stress axis. Consequently, the proactive (active) coping style, typified by aggression and territoriality, is characterised by high adrenergic (noradrenaline) axis activity and low hypothalamo-pituitary-adrenal/interrenal (HPA/HPI) axis activity. In contrast, reactive (passive) behaviour, characterised by withdrawal and immobility, is linked with a higher HPI response (De Boer et al., 1990). These dichotomous behavioural strategies associated with coping style are often, though not always, correlated with boldness (e.g. Koolhaas et al., 1999; Øverli et al., 2007).

Behavioural characteristics have a significant genetic component in many natural populations in several taxa (e.g. Álvarez and Bell, 2007; Benus et al., 1991; Fidler et al., 2007; Giles and Huntingford, 1984; Korsten et al., 2010; van Oers et al., 2004). Similarly, the physiological response to stress also appears to have a substantial underlying genetic basis. For example, it was possible to select two lines of rainbow trout, *Oncorhynchus mykiss*, for divergent endocrine response to a confinement stressor; across four generations, post-stress plasma cortisol concentrations remained significantly greater in high (HR) compared with low (LR) stress responding lines, with a moderate to high heritability (h^2=0.41–0.73) for HPI-reactivity to stress (Pottinger and Carrick, 1999; Pottinger and Carrick, 2001a). Interestingly, these lines also exhibit divergent behavioural traits which are linked with boldness: LR fish, whose behaviour shares characteristics with a bold phenotype, display longer retention of a classically conditioned response than HR fish which are considered to be relatively shy. LR fish also exhibit proactive behaviours such as enhanced aggression, social dominance, and rapid resumption of feed intake after exposure to a stressor (Øverli et al., 2007). These trout lines thus provide an excellent model to study coping style and the concomitant relationship between heritable stress responses and behavioural phenotype which is, furthermore, reflected in natural populations (Cockrem, 2007; Koolhaas et al., 1999).

Ultimately, many of these heritable differences in behaviour are manifest as differences in gene expression: a microarray analysis comparing the expression of 20,000 genes in an outbred population of *O. mykiss* highlighted ~1,000 genes which were differentially expressed in the brains of fish showing either consistently bold or shy responses to novelty (Sneddon et al., MS under review). Therefore differential gene regulation between bold and shy fish indicate that bold fish have either a different transcriptomic profile or more profoundly regulate relevant genes, and may also account for divergence of behaviour or stress physiology in these animals. If the genes identified by Sneddon and co-workers (Sneddon et al. 2005; Sneddon et al., MS under review) play a role in defining bold and shy phenotypes, they might be expected to show a different pattern of expression between HR and LR fish. With the exception of a study by Schjolden et al. (2005) there has been little examination of bold/shy behaviour within these lines of rainbow trout, nor has the possibility that behavioural variation between these lines of selected fish may be linked to discrete individual differences in brain gene expression been explored. These lines thus offer a unique opportunity to investigate the putative link between behavioural polymorphism and physiological stress responsiveness. Furthermore these aspects of animal personality and coping style can, for the first time, be correlated by quantification of the expression of a suite of candidate genes.

The broad aim of this study was to determine the extent to which neuroendocrine responses to stress, within trout selectively bred for divergent responses, correlated with bold or shy behavioural traits; we quantified this not only between the HR and LR lines but also characterised whether individual variation occurred within these lines. Further to this, the expression of a range of novel candidate genes in the brain was determined. We hypothesised (1) that LR individuals would exhibit behaviour typical of a bold phenotype and would approach a novel object more quickly and exhibit a lower stress response than HR individuals whose behaviour would resemble that of a shy phenotype, and (2) that this divergence in behavioural and endocrine responses would be associated with clear differences in the expression of genes associated with boldness (within lines) and/or the stress response (between lines).

Materials and Methods

Experimental fish

The following experiment was conducted humanely under Home Office, UK, guidelines according to the Animal (Scientific Procedures) Act 1986, and following local ethical approval. Rainbow trout, *Oncorhynchus mykiss* Walbaum, from inbred lines selected for high (HR) or low (LR) cortisol responsiveness to a standardised stressor (Pottinger and Carrick, 1999) were transferred from CEH Windermere to Liverpool where each line was held separately (~140 fish per tank) in two stock tanks (2x2x0.5 m) in a semi-recirculating system. Tanks were supplied with filtered aerated freshwater and maintained at 13±2^{o}C on an ambient 14:10 h light:dark regime. Half of the tank had an opaque overhead cover for shelter.

Fish were inspected twice daily and fed commercial pellets (Skretting, UK) at 1 % body weight per day. After a period of at least 4 months to allow fish to acclimate, trout (HR: *n*=44, 343.0±14.7 g; LR: *n*=33, 356.5±11.0 g) were selected at random from the stock tanks and placed into individual glass tanks (90x50x45 cm) which were screened from visual disturbance. All tanks were supplied with a constant flow of filtered freshwater in a semi-open system maintained at 10±1^{o}C with aeration. The trout were left to acclimate for a minimum of one week and fed daily. Experiments were conducted on fish that had resumed feeding after this period.

Behaviour

A custom-built low-light video camera was situated in front of the tank and a second camera placed to the side of the tank. Measuring rulers (0.5 cm intervals) were arranged horizontally and vertically along the front of the tank to measure proximity of the fish to the novel object. The fish were allowed 10 minutes to acclimatise to the potential disturbance arising from setting up the cameras. Behaviour of the fish without disturbance was then recorded for 10 minutes, before a novel object was added. The novel object test is a standard paradigm to differentiate between bold and shy individuals (Wilson et al., 1993). The novel object was placed as near to the centre of the tank as possible, and the behaviour of the fish was recorded for a further 10 minutes after which the object was carefully removed. This test was repeated a week later to assess the level of consistency of behaviour displayed by the experimental individuals. Novel objects were varied between trials to ensure the fish did not become habituated to a familiar shape, and included an orange frustum-shaped bung (7.05 cm mean diameter, 4.9 cm height) and a bipyramidal Duplo$^{(R)}$ construct (height 13.5 cm, and maximum widths 7.6x6.3 cm) of black, red and blue bricks.

Scoring of the behaviour was accomplished using custom designed behavioural analysis software. Three measurements each of three separate behaviours were initially scored based on the activity levels of the subject and its proximity to the novel object (Table 1; see Frost et al., 2007). Principal components analysis (Minitab ver.15.1) was subsequently used to identify the key behaviours that differentiated bold fish from shy. Latency to approach within 5 cm (s) of the object was strongly represented in the first principle component (eigenvalue=3.53, loading for 5 cm latency=-0.41) and could be solely used to differentiate between bold and shy groups. This measure has previously been used to identify boldness in fish (Coleman and Wilson, 1998; Frost et al., 2007). Loadings for six of the measurements were well represented in the first principal component, and two of these, frequency of entering a 10 cm zone (min^{-1}) centred on the object (loading=0.459) and duration (s) spent passive (loading=-0.381), were selected for further analysis. Passive behaviour was defined to exclude swimming (movement of the fish generated by propulsion using the fins, of no less than approximately one body length) but include drifting, fish pivoting on their own axis, any minor movements made to maintain position, and resting on the bottom of the tank.

Hormone analysis and quantification of gene expression

Subsequent to, and on the same day as, the final behavioural trial, approximately half of the fish (*n*=34) were netted and exposed to air for 60 s to induce an acute physiological stress response before being placed back into their tank (Pickering and Pottinger, 1989). Fifteen minutes after emersion, the trout were netted again before being killed humanely by concussion. To obtain unstressed plasma cortisol concentrations, fish were killed by concussion without this treatment. Individuals were killed at the same time each day to ensure that interpretation of differences in hormone levels was not compromised by diel fluctuations in plasma cortisol (Pickering and Pottinger, 1983). Immediately after euthanasia, a 2 ml blood sample was taken from the caudal vessels using sterile 25 g needles and heparinised 2ml syringes. The supernatant plasma was aspirated, divided into aliquots and frozen at -20°C. Plasma cortisol levels were determined by radioimmunoassay (Pottinger and Carrick, 2001a).

Immediately following blood sampling, the whole brain was removed and stored at -80°C until RNA extraction, and fish were sexed. Total RNA was extracted from trout brain using TRIzol® (Invitrogen Life Science, UK), with RNA eluted into 50 µl RNase-free water. RNA concentrations were determined by optical density at 260 nm using a NanoDrop ND-1000 spectrophotometer (LabTech International, UK) system and the quality of the samples assessed by 2 % agarose gel electrophoresis. For each sample, approximately 1 µg of mRNA was reverse-transcribed into first-strand cDNA using random hexamers and SuperScript™ III reverse transcriptase (Invitrogen Life Science, UK), following the manufacturer's protocol.

The candidate genes selected for this study were chosen for their roles in behaviours associated with boldness, such as aggression, anxiety and memory, or for their association or direct involvement with the stress response (Table 2). Furthermore, six of these genes, ependymin, $GABA_A$, calmodulin, MHCI, Hbα4, and a lipocalin, retinol binding protein, were differentially regulated between bold and shy rainbow trout in a previous study (Sneddon et al., 2005; Sneddon et al., MS under review). Eight pairs of primers for these genes were developed using Primer Express® 3.0 software against *O. mykiss* sequences (Table 3). For RT-PCR, ~0.05 µg of the cDNA was amplified in a 10µl PCR (using 5 µl Fast SYBR Green, Invitrogen Life Science, UK) primed with 2pmol each primer. Thermal cycling conditions, using a 7500 Fast Real-Time PCR System (Applied Biosystems), were: 10 min at 95°C, followed by 40X [95°C 3 s, 60°C 30 s] and then [95°C for 15 s, 60°C for 60 s, 95°C for 15 s and 60^{o}C for 15 s], which allowed the construction of a melting curve to assess the specificity of the product.

Data Analysis

None of the data were normally distributed (Anderson-Darling; Minitab, ver.15.1) and thus non-parametric tests were applied. These tests also reduce Type 1 errors since there were unbalanced sample sizes due to unequal numbers of bold and shy fish in each line. A Wilcoxon Signed Rank Test was used to analyse the difference between behavioural scores of the first and second trial to test for consistency in latency to approach within 5cm of the novel object (Minitab, ver.15.1). Subsequently, data were separated for trout showing consistently bold (approach to 5 cm of the object within 180 s in both trials; *n*=28) or shy (do not approach to 5 cm within 300 s in both trials; *n*=13) behaviour. Scores for each of the behaviours were then averaged over the two trials and compared between bold and shy groups within both the HR and LR line using Mann-Whitney U-tests (R, ver.2.7.0), including sequential Bonferroni treatment (Rice, 1989) for multiple tests.

Plasma cortisol concentrations for stressed and unstressed trout were compared between the two stress lines (unstressed: HR *n*=13, LR *n*=23; stressed: HR *n*=27, LR=7), between consistently bold and shy trout (unstressed: bold *n*=12, shy *n*=5; stressed: bold *n*=14, shy *n*=7) and between sexes (female *n*=17, male *n*=15) using Mann-Whitney U Tests (R, ver.2.7.0). For RT-PCR, cycle threshold (Ct; the first cycle number at which fluorescence is significantly greater than background levels) and efficiency values for each gene were exported into REST (ver.2.0.7; Pfaffl et al., 2002) whereby the relative expression of each gene between bold and shy fish or between fish from each of the two stress lines, normalised to a reference gene (GAPDH), was calculated. Statistical analysis was subsequently accomplished through REST's bootstrap randomisation procedure.

Results

In unstressed rainbow trout, (Fig. 1A) plasma cortisol concentrations were significantly greater in LR fish compared with the HR line (3.16 and 1.34 ng ml^{-1} respectively; W=47.0, p<0.01, n_1n_2=23,13), with no significant difference between sexes (W=89.0, p=0.15, n_1n_2=17,15). By contrast, after exposure to a stressor, HR trout had a greater plasma cortisol response than did LR fish (67.42 ng ml^{-1} and 27.14 ng ml^{-1} respectively; W=158.0, p<0.01, n_1n_2=27,7; Fig. 1B), and while blood-cortisol concentrations were higher in female trout (73.53 ng ml^{-1}) than in males (46.36 ng ml^{-1}), the response was highly variable so insignificant (W=177.0, p=0.06, n_1n_2=17,15).

Consistent with other studies, boldness showed a bimodal (i.e. u-shaped) distribution and tended towards extremes in individual trials both as a group (Fig. 2A) and separated by line (Fig. 2B, 2C), with fish exhibiting clear bold (approaching 5 cm of the object within 60 s; n=63) or shy (not approach within 5 cm during the trial; n=42) behaviour. Individual trout were consistent in their latency to approach within 5 cm of a novel object over two trials (W=913.0, p=0.113, n=77), thus confirming the utility of this measure. Rather than being associated predominantly with one or other line, both bold and shy fish were identified within each line. Moreover, there was a tendency for fish to be bold rather than shy in both lines (Fig. 3); although there were proportionately more shy fish in the HR line compared to the LR line (15:9 bold and shy compared to 13:4 bold and shy respectively), this difference was not significant (χ^2_1=0.891, p=0.344). Furthermore, although plasma cortisol concentrations profoundly differed between the two lines, there was no significant difference observed in cortisol concentration between bold and shy fish, regardless of whether they were unstressed (W=37.0, p=0.51, n_1n_2=12,5; Fig. 1A) or stressed (W=89.0, p=0.15, n_1n_2=17,15; Fig. 1B).

Although bold and shy fish could be distinguished within each line by their approach latency to within 5 cm of a novel object, trends in other behaviours were apparent in HR trout but not in LR fish. Within the HR line, consistently bold fish spent less time overall being passive (W=244.5, p<0.01, n_1n_2=15,9; Fig. 4A) than shy trout but this was not true of trout from the LR line (W=103.0, p=0.126, $n1n2$=13,4). Similarly, bold HR trout also entered the 10 cm zone about the object more frequently (W=138.0, p<0.01, n_1n_2=15,9; Fig. 4B) than shy fish, but no significant difference was detected between bold and shy fish in the LR line after Bonferroni treatment for multiple tests (W=135.0, p=0.048, $n1n2$=13,4).

Differences between the stress lines were evident in the relative expression levels of six candidate genes: ependymin, calmodulin, MHCI, $GABA_A$, vasotocin and RBP were significantly upregulated in the brains of LR fish compared with HR fish (Table 4; Fig. 5B). Average fold change varied from an upregulation factor of 1.89 for AVT up to 5.92 for MHCI. In contrast, expression of both POMC and Hbα4 were almost identical between the lines. However, bold and shy fish, independent of selection line, did not significantly differ in the expression levels of any of these genes, with the expression of most genes marked by large variance due to pooling of samples within the stress lines (Fig. 5A).

Discussion

Boldness is a complex behavioural trait that has previously been associated with coping style (Koolhaas et al., 2007), and may thus be assumed to correlate with the magnitude of the physiological stress response. In this study, bold and shy rainbow trout were identified within distinct stress-response lines of rainbow trout by measuring their behavioural response to novelty: this is the first characterisation of both bold and shy phenotypes within these lines. Whilst divergent plasma cortisol responses to a stressor were evident between the HR and LR lines, consistent with earlier findings (summarised in Øverli et al., 2005), no significant relationship between boldness and stress responsiveness was found either between or within lines. Although a slightly larger proportion of LR trout exhibited a bold phenotype than HR trout this was not significant and no associated differences were observed in post-stress plasma cortisol levels between bold and shy individuals independent of selection line. Similarly, physiological divergence between the HR and LR lines was correlated with differences in regulation of six candidate genes in the brain, but bold and shy fish did not exhibit any dissimilarity in the regulation of these candidate genes.

Differences between HR and LR lines

The clear bimodal response to novel objects and the frequency of bold and shy fish within line and as a whole were similar to those observed in outbred rainbow trout (Frost et al., 2007). Boldness thus apears to be bimodally distributed in this species, a response seemingly maintained even in lines selected for divergent responsesiveness to a stressor. Other species may exhibit different distributions, such as a normal distribution with relatively fewer bold and shy compared to intermediate fish in pumpkinseed sunfish (Wilson et al., 1993). Thus bold/shy distributions may reflect interspecific or between-population differences in intrinsic factors or extrinsic pressures that may drive variation in personality. Even rearing conditions can cause a prevalence of certain behavioural types within a population of salmonid fish (Sundström et al., 2004).

The consistent divergence in the HPI reactivity to stress between the two stress lines is in accordance with earlier studies on these selected lines using confinement to induce a stress response (Pottinger and Carrick, 1999; Schjolden et al. 2005). However, the equally strong divergence among some genes involved in the stress response has not previously been demonstrated and emphasises the strong genetic basis that underpins stress physiology in rainbow trout (e.g. Pottinger and Carrick, 1999; Pottinger and Carrick, 2001a) and possibly other vertebrates (Yao and Denver, 2007). Further work should focus on determining whether these responses are consistent throughout the entire pathway or whether genetic regulation occurs only at key loci within the response. In unstressed fish plasma cortisol concentrations were higher in LR fish than in HR fish, the reverse of an earlier observation in these lines (Pottinger and Carrick, 2001b), and may reflect factors responsible for modulation of the unstimulated HPI axis that have yet to be identified in fish.

Differences in whole-brain gene expression between the stress lines represent the first evidence that the key phenotypic difference between the lines, divergence in stress responsiveness, is reflected in a broader suite of correlated molecular responses linked with boldness or stress physiology. Immune function can be compromised by chronic stress possibly explaining why MHC, CaM and RBP were each upregulated in LR fish relative to HR fish, since the corresponding proteins are associated with the immune system or response. The Ca^{2+}/CaM complex directly or indirectly controls a number of mechanisms and enzymes involved in the immune response, including aspects of the MHC and the serine-threonine kinases CaMK I, II and IV (Racioppi and Means, 2008). RBP meanwhile has been implicated in inflammatory processes associated with immune responses (Flower, 1996). Low stress-responding animals are often characterised as having improved health over those with a high response, and a major issue associated with sustained elevation of cortisol is a reduction in immunocompetence and increased susceptibility to pathogens (Wendelaar Bonga, 1997). Some aspect of divergent immunological parameters between low and high stress responders thus appears to be controlled at the molecular level; divergence in gene expression, particularly that of proinflammatory genes, has been identified between stress coping styles (MacKenzie et al., 2009) and may reflect differences in circulating steroid concentrations.

Both $GABA_A$ and AVT genes were upregulated in LR fish, and changes in expression of both genes have been related to aggressive behaviour (Backström and Winberg, 2009; Miczek et al., 2003), a defining characteristic of stress coping styles and also of these stress lines, where LR trout are more aggressive (Pottinger and Carrick, 2001a). However, high levels of AVT tend to inhibit aggression in territorial teleosts such as rainbow trout (Backström and Winberg, 2009), so higher expression of AVT in LR trout is seemingly paradoxical and merits further investigation. Backström and Winberg (2009) suggest that the aggressive output influenced by AVT could be mediated by other systems, in particular the brain serotonergic system, and thus studies that evaluate serotonergic activity together with AVT concentration or expression may throw light on these observations.

Expression of POMC may not differ between subjects with different stress-coping abilities (Centeno et al., 2007), but rather physiological variation in the HPI axis may occur downstream during post-translational modification, or via differences in target tissue sensitivity, and this may indeed be the case for the HR and LR trout lines. Concentrations of adrenocorticotropic hormone (ACTH) in the blood of HR and LR fish did not differ significantly during stress; instead, the responsiveness of the interrenal to ACTH differed between the lines (Pottinger and Carrick, 2001b), and a similar process may operate here.

Physiology and boldness within the lines

The results suggest that the distribution of bold and shy individuals within each line was not consistently influenced by the selection process despite evidence from earlier studies that the two lines differ consistently in certain key behavioural traits (Pottinger & Carrick, 2001; Øverli et al., 2005, 2007). Furthermore, within the HR line the existence of a behavioural syndrome was evident where boldness was significantly linked with activity levels, suggestive of risk-taking and risk-averse strategies in bold and shy fish respectively (Sneddon, 2003). Indeed the bold fish in this study were characterised by making more use of the available tank space and making less effort to avoid the object. In contrast, a clear behavioural syndrome was not apparent in the LR line. Behaviour of shyer fish within the LR line perhaps was not as well defined compared to natural populations (e.g. Wilson et al., 1993), which may reflect the generally more bold or proactive coping style exhibited by low stress-responding animals (Koolhaas et al., 1999). Alternatively, coping style theory predicts that proactive animals are more rigid in behaviour whereas reactive animals are flexible (Koolhaas et al., 1999), which could suggest they are able to draw on a greater pool of behaviours when reacting to environmental stimuli. These LR and HR trout may be exhibiting these same trends, where LR animals may simply have a less diverse or more limited behavioural repertoire. However, a particularly low sample size for consistently shy fish in the LR line, although originally expected considering previous theory regarding behaviour in LR animals, may limit the power to draw robust conclusions. Nonetheless previous studies have been unable to conclusively link novelty-induced boldness with stress physiology (e.g. Schjolden et al., 2005); our data indicate that this is due to both bold and shy phenotypes existing amongst low and high stress-responding groups.

The absence of a well-defined link between cortisol levels and boldness within the lines was surprising given previously observed correlations between the magnitude of the stress response and behaviour (Koolhaas et al., 1999; Øverli et al., 2005). Both boldness and shyness were represented within each selected line, and so the correlations between stress responsiveness and behaviour or boldness that have previously been reported (e.g. Øverli et al., 2007; Schjolden et al., 2005) are not always observed. One reason may be that if boldness is context-specific individual behaviour will vary dependent upon the situation (e.g. in familiar compared to unfamiliar environments; Schjolden et al., 2005). This would potentially confer adaptive advantages particularly in an inconsistent environment (Bell, 2007; Coleman and Wilson, 1998; Wilson and Stevens, 2005). Such variation may be elicited by the type or severity of the stressor or by familiarity with the test environment (Brelin et al., 2008; Misslin and Ropartz, 1981; Schjolden et al., 2005). Contrasting behavioural responses observed between studies may additionally arise from variation in methodological approach to characterising boldness. Furthermore, Schjolden et al. (2005) could not find consistent differences in behavioural responses between HR and LR rainbow trout across several tests including the response of the subjects to a novel object, which may be a result of comparing average behaviours between the lines rather than characterising boldness within each line as in the present study. Thus whilst aggression, a defining component of coping styles and a putative element of boldness, may strongly and consistently correlate with HPI axis reactivity the same is not necessarily true of responses to novelty. It therefore seems apparent that boldness may not directly correlate with stress coping style, and future studies should explore the extent to which the stress response is linked with behavioural phenotype. However, there is a need for standardisation in protocol to determine the degree of boldness and which features of an individual’s behavioural repertoire are dependent on or act congruously with hormonal stimulation under greater homeostatic threat.

Alternatively, the existence of bold and shy phenotypes within line instead of correlating with stress responsiveness suggests that coping style theory (Koolhaas et al., 1999) may simply not be true in all cases. Here, we provide novel data to suggest that divergent personality traits persist within a population or species irrespective of stress coping style. Experience, brought about by environmental or social influences, can shape an individual's behavioural strategy (Brown et al., 2007; Frost et al., 2007). Moreover, behavioural variation can occur within a group regardless of genetic background, and when environmental conditions are identical for each individual (Metcalfe et al., 1989). With this in mind, it is not surprising that this study and other recent work have highlighted the complexity inherent in the genetic control of personalities (Korsten et al., 2010). Our data reinforce this, since, despite previous studies that identified different gene expression profiles between outbred rainbow trout with different behaviours (e.g. dominance, Sneddon et al., 2005; boldness, L.U. Sneddon, MS under review), no such divergence between bold and shy fish was uncovered in this study. Gene expression may vary between discrete regions of the brain (Bernier et al., 1999; Feldker et al., 2003; Larson et al., 2006), and can relate directly to behavioural differentiation (Greenwood et al., 2008), and thus a single measurement encompassing all brain regions could obscure more fine-scale differences in expression. Thus, whilst no difference in expression of the studied genes was found across the entire brain, that is not to say that bold and shy individuals express these genes in different localised areas of the brain: whilst differential expression of these genes between the stress lines was profound, variation amongst bold and shy groups may be more subtle. It is of course possible that the lines lack genetic diversity, or that different genes may be involved in the expression of bold/shy behaviour. However, the clear divergence in expression of some of the examined genes in a previous study (LU Sneddon, MS under review) suggests the latter not to be the case, but does emphasise the complexity of bold and shy personalities in rainbow trout. Given that the expression of boldness was independent of selection line, it is likely that the genetic control of boldness may be unrelated to the controlling divergent elements of the selected stress response.

Conclusions and Implications

The results of this study indicate a complex relationship between stress responsiveness and behaviour in the HR and LR lines of rainbow trout. Stress responsiveness is a heritable trait in trout (Pottinger and Carrick, 1999; Pottinger and Carrick, 2001a) and the present study demonstrated that divergence in stress responsiveness correlates with differential expression of six novel candidate genes with functions in relevant behaviour and physiology. However, contrary to our hypothesis, the physiological and gene expression responses evident in the selected HR and LR lines did not correlate with boldness or shyness, traits that were identified in substantial numbers within each line. This suggests that the adoption of these contrasting behavioural strategies may not be explained entirely by genetic background or stress coping style and may instead be influenced by external factors that should be considered in theoretical and empirical studies. Experience and environmental influences may cause quite distinct changes in behavioural responses throughout an animal's life history (Frost et al., 2007; Ruiz-Gomez et al., 2008), which may result in behavioural polymorphism even within coping styles. Therefore, it is important for future studies to take into account of how experience and external factors may mould boldness. This may explain why variation in these behavioural phenotypes persists in natural populations to ensure a proportion of individuals can adapt to and survive any perturbations.

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Table 1: The definition and measurements recorded of the behaviours assessed during the novel object tests in rainbow trout, *Oncorhynchus mykiss*.

Behaviour	**Definition and measures**
Within 5cm	The subject was within a delineated zone extending to 5cm around the object. Three measurements were taken: 1) **latency**, the time (s) taken to enter this zone for the first time; 2) **duration**, the total time (s) spent within this zone; 3) **frequency**, how often the subject entered this zone.
Within 10cm	The subject was within a delineated zone extending to 10cm around the object. Three measurements were taken: 1) **latency**, the time (s) taken to enter this zone for the first time; 2) **duration**, the total time (s) spent within this zone; 3) **frequency**, how often the subject entered this zone.
Passive	Inactivity; includes drifting, minor movements to maintain position within the tank, pivoting on its own axis and resting on the bottom of the tank, but excludes swimming. Three measurements of passive behaviour were recorded: 1) **latency**, the time taken (s) to begin displaying passive behaviour; 2) **duration**, the total time the subject spent (s) displaying passive behaviour; 3) **frequency**, how often the subject displayed passive behaviour.

Table 2: Genes (including abbreviations and known major functions) used in this study. Italicised genes showed differential expression between bold and shy rainbow trout, *Oncorhynchus mykiss*, in a previous microarray study (Sneddon et al. 2005; LU Sneddon, MS under review).

Gene	**Abbr.**	**Functions**
Ependymin	Epd	Memory/learning[1]; Cold tolerance[2]; Regeneration[3]
γ-Aminobutyric acid A	GABA_A	Anxiety[4]; Aggression[5]; Memory[4]
Calmodulin	CaM	Calcium binding (Memory[6]; Nerve growth[6]; Immune system[7])
Major histocompatability complex Class I	MHC I	Immune system[8]; Kin recognition[8]
Haemoglobin α4 subunit	Hbα4	Oxygen transport
(Arginine) vasotocin	AVT	ACTH secretion[9]; Modulation of social and non-social behaviour[9]
Proopiomelanocortin	POMC	Stress response[10]
Retinol binding protein	RBP	Vitamin A transport[11]; Stress/Immune response[12]

[1] Shashoua (1991), [2] Tang *et al*. (1999), [3] Suárez-Castillo *et al*. (2004), [4] Kalueff and Nutt (1997), [5] Miczek *et al*. (2003), [6] Stevens (1983), [7] Racioppi and Means (2008), [8] Götze (1977), [9] Goodson and Bass (2001), [10] Winberg and LePage (1998), [11] Goodman (1980) [12] Flower (1996).

Table 3: Primer sequences for RT-PCR for eight genes implicated in behavioural responses, and for a reference gene (*), including accession number (where primers were generated from a single sequence), and amplicon size and melting temperature, T_m. Primers were developed using Primer Express® 3.0 software, and were diluted to a working concentration of 10 pmol μl^{-1}.

Gene **Accession No.**	**Forward (5’ – 3’)**	**Reverse (5’ – 3’)**	**Size (bp)**	**T_m (°C)**
Ependymin NM_001124693	CTC ATG CTC ACG CTC TGG AA	CCA AAA ACA GCT CAA CCT GAT G	60	83
GABA$_A$ BT073523	CTC ATC CGA AAG CGA ATC CA	CAC ACT CTC GTC ACT GTA GG	156	81
Calmodulin	CCG GGA GGC TGA TAT CGA T	CGT CAT CAT CTG CAC AAA TTC TTC	64	81
MHC1	AGT CCC TCC CTC TGT GTT TCT G	TCG CGT GGC AGG TCA CT	62	62
POMC NM_001124718	AGC GCT ATG GAG GGT TCA TG	CAA CGT GAG CAG TGG TTT CTG	62	82
Hbα4 BT074353	GAA GAA GCG CGG CAT CAC	TCG TCC ATG TGG CCA ACA	60	81
AVT DQ291141	ACC CAG CGG TCC TAT ATT ATG ATC	GGC ATG CTG AGG ACC AGA CT	62	81
RBP NM_001124278	GGA CAA TGT CGT CGC TCA GTT	CGT GGG CAG TTG CAG TCA	62	80
*GAPDH** AF027130	TGT TGT GTC TTC TGA CTT CAT TGG	CCA GCG CCA GCA TCA AA	60	81

Table 4: Relative expression (normalised to a control gene, GAPDH; RE) and *p* values for the comparisons of expression of eight genes, selected for implicated roles in boldness, between bold and shy or between high (HR) and low (LR) stress responsive rainbow trout, *Oncorhynchus mykiss*. Asterisks denote significant difference between the groups (REST, in Pfaffl et al., 2002): *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

	Boldness		**Stress Line**	
	RE	***p***	**RE**	***p***
Epd	0.82	0.52	2.63	***
MHC I	0.69	0.46	5.92	***
CaM	0.75	0.31	2.09	**
$GABA_A$	1.02	0.96	1.93	**
POMC	1.03	0.98	0.76	0.70
Hbα4	0.94	0.88	0.99	0.98
AVT	0.90	0.72	1.89	*
RBP	0.80	0.42	2.01	**

Figure 1: Median plasma cortisol (ng ml^{-1}; ± 90^{th} and 10^{th} percentiles) in unstressed (A; *n*=36) and stressed (B; *n*=34) rainbow trout, *Oncorhynchus mykiss*. In each case, comparisons were made between high (HR) and low (LR) stress responsive lines, between individuals determined bold and shy by a novel object test, and by sex. Asterisks denote significant difference between groups (Mann-Whitney test): **, $p<0.01$; ***, $p<0.001$.

Figure 2: Frequency of individual trials in which individual rainbow trout, *Oncorhynchus mykiss* (*n*=154), either (A) as a whole (*n*=154) or separated into (B) the HR (*n*=88) or (C) the LR (*n*=66) stress lines, approached within 5 cm of a novel object within a certain period of time (*n*=154).

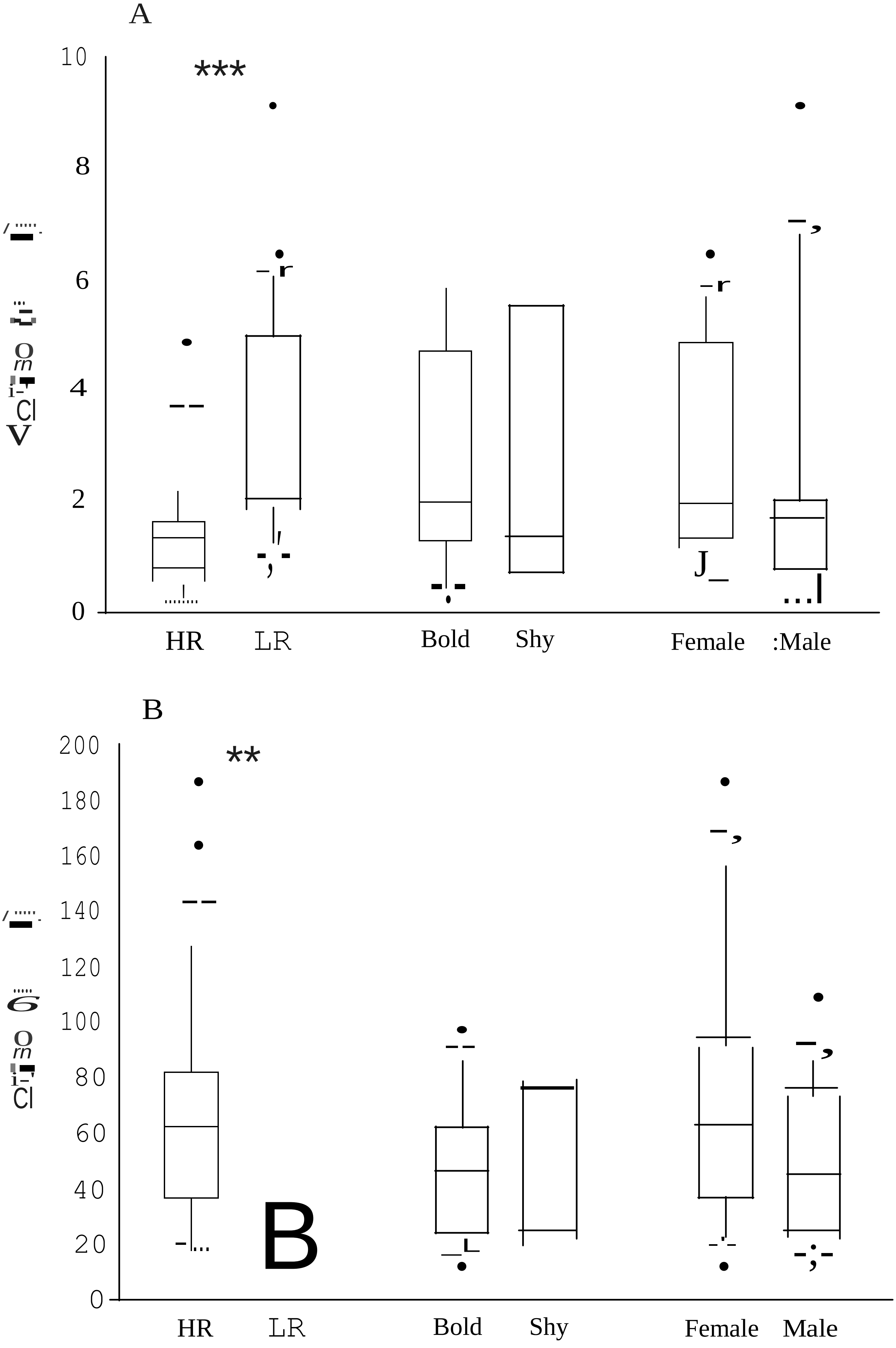
Figure 3: Percentage of rainbow trout, *Oncorhynchus mykiss*, showing consistently bold (white) or shy (grey) behaviour in lines bred for high (HR; *n*=24) and low (LR; *n*=17) cortisol response to stress, and in both groups combined.

Figure 4: Median (± 90^{th} and 10^{th} percentiles) (A) duration of passive behaviour and (B) frequency of approaching to within 10 cm of a novel object for bold and shy rainbow trout, *Oncorhynchus mykiss*, within the HR (white; *n* for bold=15, *n* for shy=9) and LR (grey; *n* for bold=13, *n* for shy=4) stress lines. Asterisks denote significant difference between groups (Mann-Whitney test): **, $p<0.01$; ***, $p<0.001$.

Figure 5: Median relative expression ($\Delta Ct_{\mathrm{reference}}$ - $\Delta Ct_{\mathrm{target}}$; ± 90^{th} and 10^{th} percentiles) of eight candidate genes compared between (A) bold (*n*=28; white) and shy (*n*=13; grey), and (B) high (HR; white; *n*=22-25) and low (LR; grey; *n*=17) stress responding rainbow trout, *Oncorhynchus mykiss*. Epd = Ependymin; MHCI = major histocompatability complex I;

CaMcalmodulin; GABAy-Aminobutyric acid A; POMCproopiomelanocortin; Hba4 haemoglobin u4 subunit; AVT vasotocin; REP **retinol** binding protein. Asterisks denote significant difference between the groups (REST, in Pfaffi eta!., 2002): *, *pSO.OS;* **,pSO.Ol; ***,pSO.OOI.

5-Figure 1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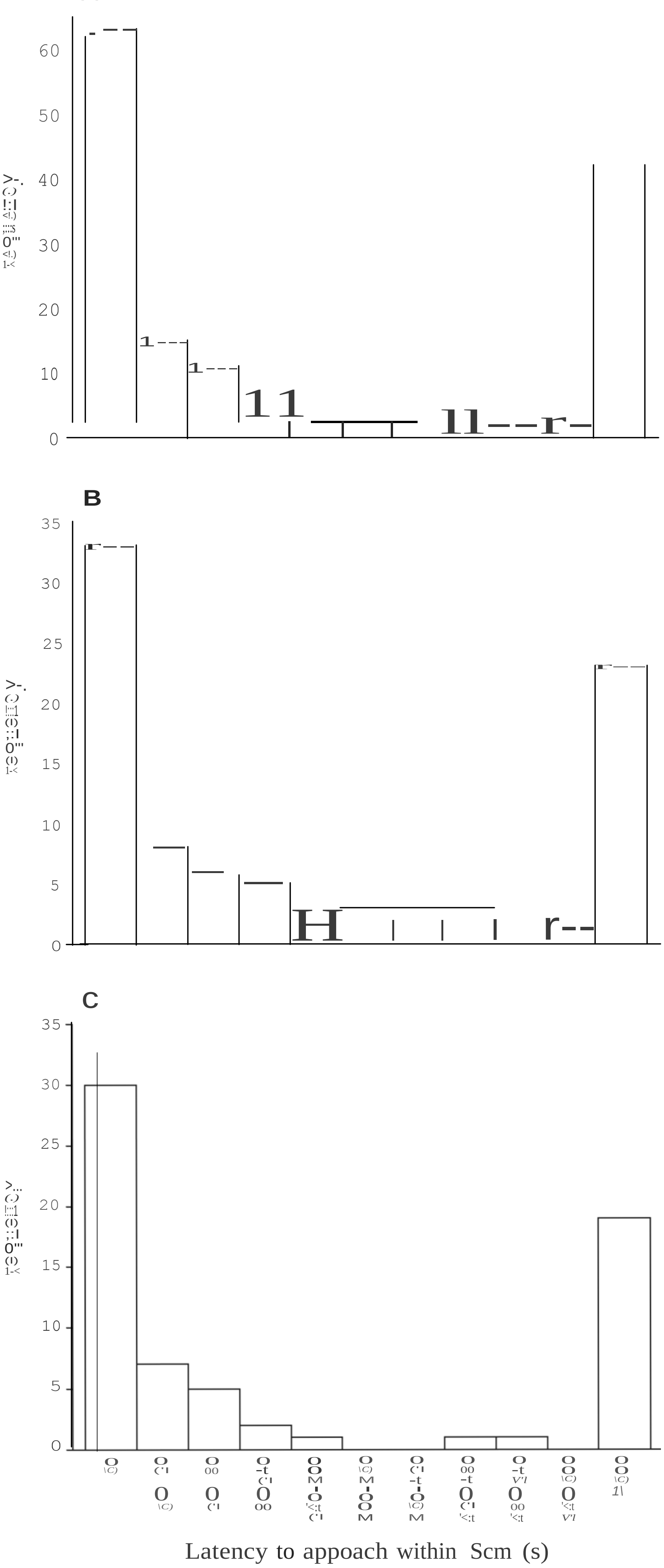
5-Figure 2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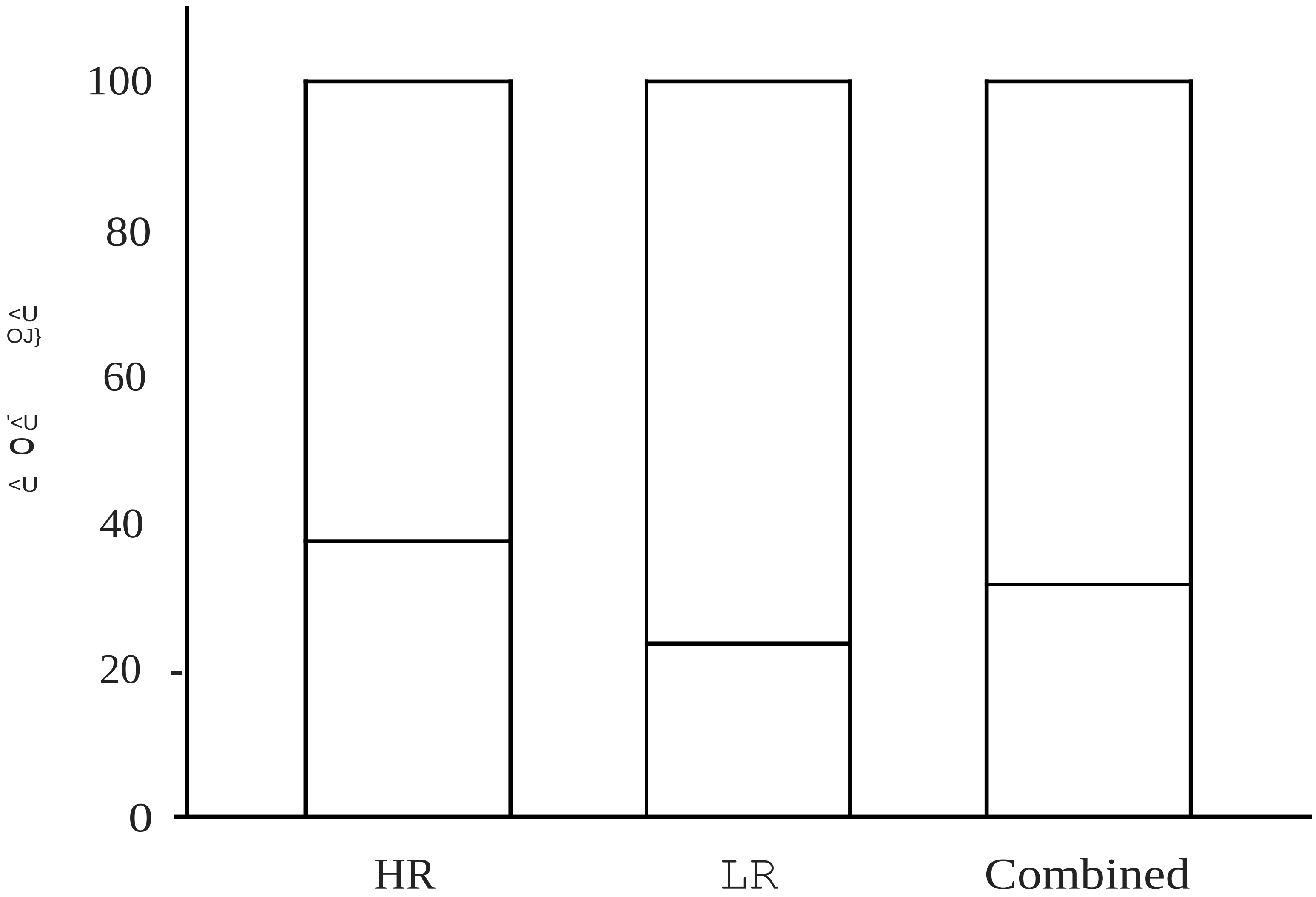
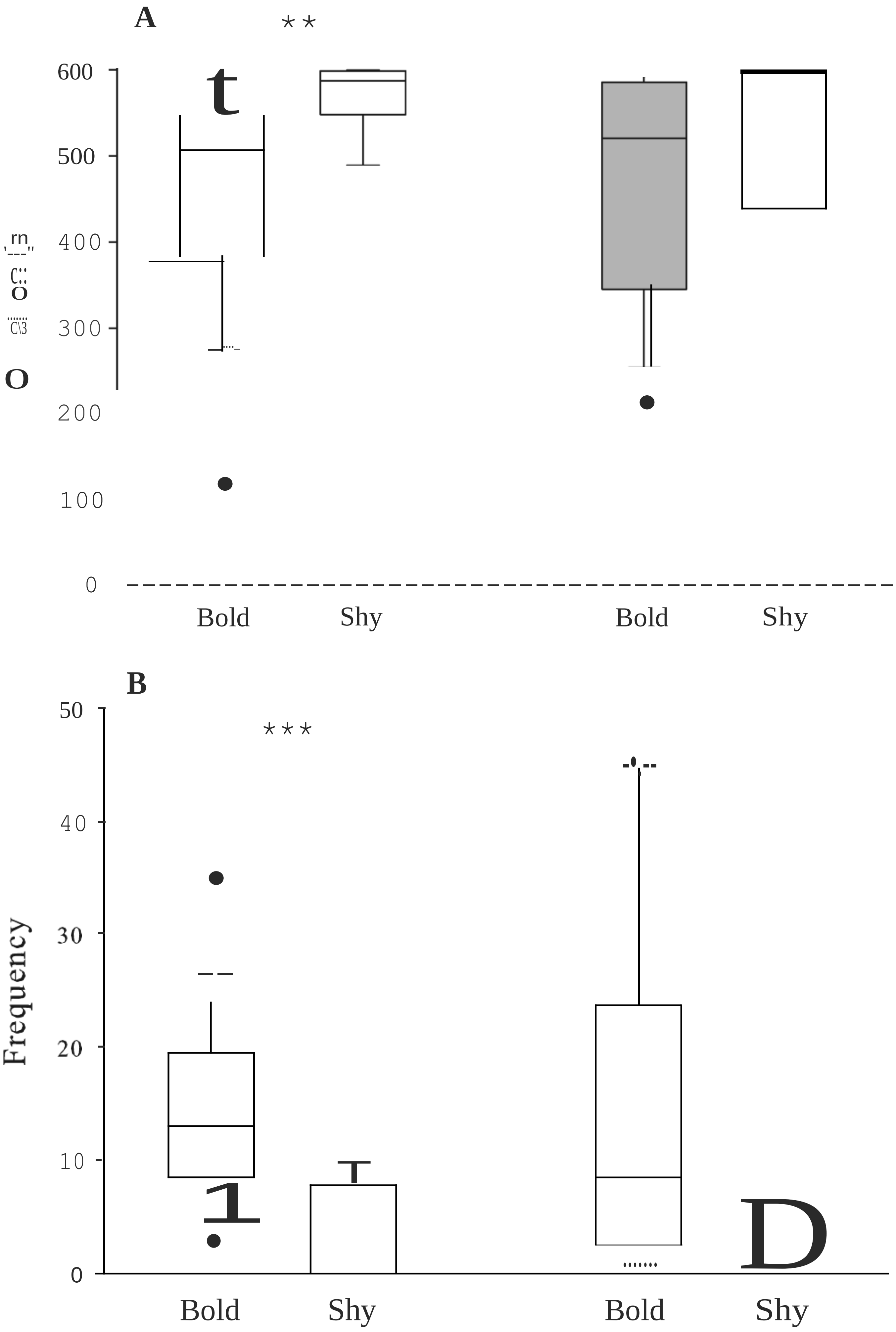
Latency to appoach within Scm (s)

5-Figure 3



5-Figure4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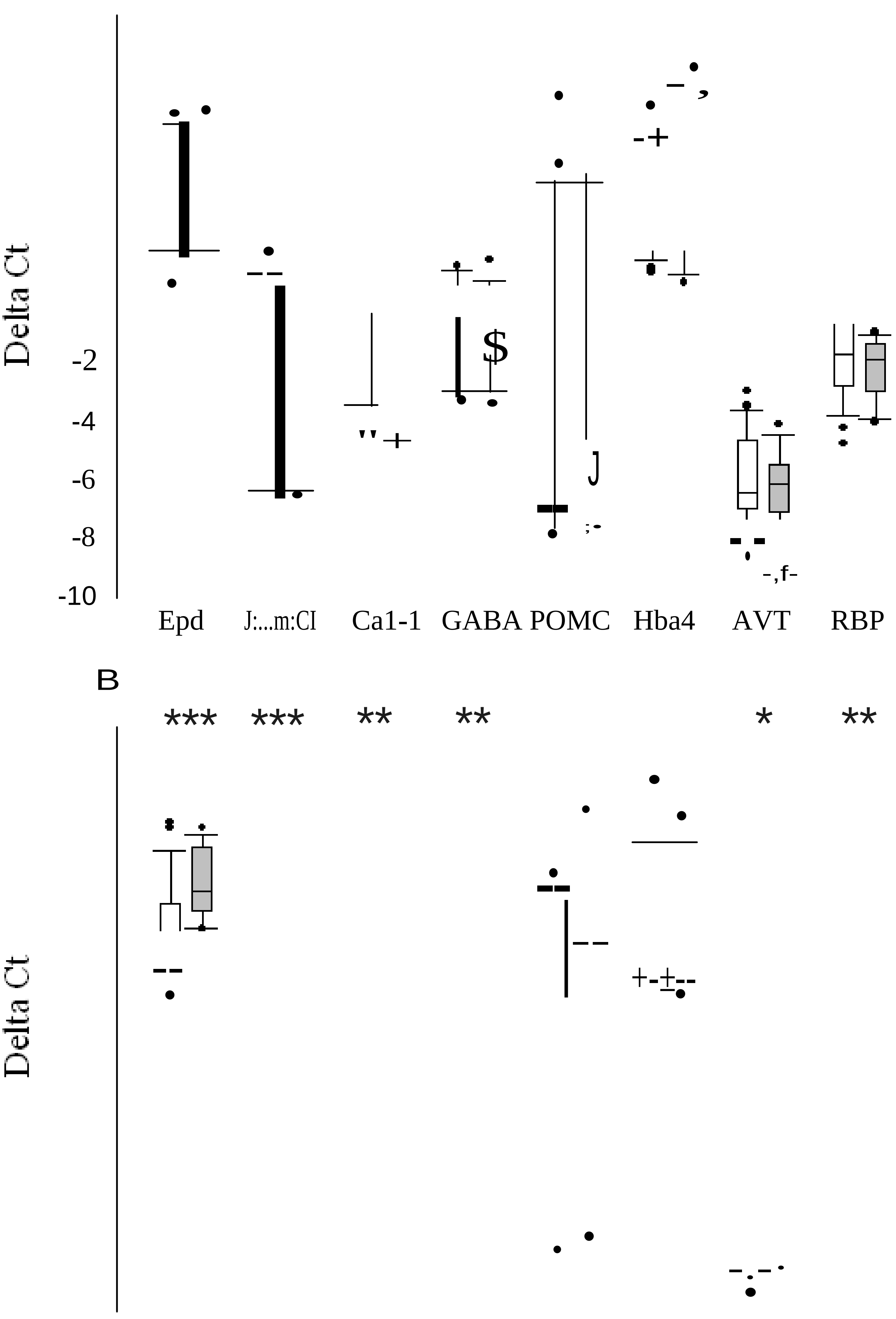
5-Figure 5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