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Effects of Forced Alcohol Drinking on Alcohol-Water Choice in Three Pairs of Rat Lines Selectively Bred for Differences in Alcohol Preference

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Abstract

Three pairs of Indiana University rat lines (P/NPs, HAD1/LAD1s, and HAD2/LAD2s) have been bred in the School of Medicine colony to drink high vs. low daily amounts of a 10% v/v alcohol test solution (>5.0 g/kg/bw vs. <1.5 g/kg/bw), and a high vs. low proportion of alcohol to water (>2:1 vs. <0.5:1), by the end of a 3-week alcohol-water choice condition. The choice phase is always preceded by 4 days of a forcing procedure with alcohol as the only fluid. The present study examined the contribution of this forcing procedure to the alcohol intake of animals in each pair of lines by comparing daily alcohol intake of rats housed in experimental chambers for a Forced group (4 days with only alcohol solution to drink followed by 22 choice days) versus a Choice group (both alcohol and water available all 26 days). As expected, under the initial alcohol exposure, *high-drinking line* rats drank more alcohol than *low-drinking line* rats, and all Forced groups drank more alcohol than Choice groups. At the start of the choice phase, all *low-drinking line* Forced groups immediately dropped their alcohol intake to the level of their Choice groups. In contrast, all *high-drinking line* Forced groups maintained a high level of alcohol intake under choice, while all *high-drinking line* Choice groups slowly increased average alcohol intake across the 22-day choice phase, ending near the average intake of the Forced animals. However, a small subset of *high-drinking line* Choice animals failed to increase alcohol intake until subsequently forced with alcohol for 4 days and retested in choice. These results indicate that the alcohol forcing procedure used in deriving these lines has resulted in the selection of multiple pathways to a high-drinking phenotype. In addition, *high-drinking line* animals appear more sensitive to difference between laboratory and colony testing environments than *low-drinking line* animals. Our data suggest that the high-drinking lines may represent an appropriately complex model of multiple factors contributing to the genesis of human alcoholism.

Keywords

forced alcohol exposure; high- and low-alcohol-drinking phenotypes; selection

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Introduction

Important animal genetic models of human alcoholism have been developed by selectively breeding pairs of high- and low-alcohol drinking rat lines. Three such pairs of lines have been bred and maintained at the Indiana University School of Medicine colony in Indianapolis: Inbred Alcohol Preferring and Non-Preferring rat lines (P and NP), and two replicate pairs of genetically diverse High and Low Alcohol Drinking rat lines (HAD1 & LAD1, and HAD2 & LAD2) (Li et al., 1993; McBride and Li, 1998; Murphy et al., 2002). To meet the combined selection criteria for breeding in each high-drinking line, individually housed colony rats, by the end of 3 weeks of alcohol water choice, must ingest more than 5.0 g/kg/day of a 10% (v/v) alcohol solution and show a 2:1 ratio of alcohol to water intake. Under the same circumstances, the breeding criteria for low-alcohol drinking lines requires ingestion of less than 1.5 g/kg/day of alcohol and a 0.5:1 ratio of alcohol to water (Li et al., 1993; McBride and Li, 1998; Murphy et al., 2002).

A potentially important, although not closely examined, component of the standard selection procedure for high- and low-drinking Indiana lines is that the rats are exposed to 4 days of forced alcohol (without water access) just prior to testing in the 3-week alcohol-water choice phase. The primary question addressed in this research was to what extent this 4-day forcing procedure, when alcohol is presented as the only fluid, affected subsequent expression of high- and low-drinking genotypes in the Indiana P/NP, HAD/LAD1, and HAD/LAD2 pairs of lines? For example, is forcing alcohol irrelevant, a facilitator, or a necessary condition to obtain typical alcohol intake in these lines? An additional question was to what extent repeated exposure to alcohol in the alcohol-water choice phase, whether preceded by forcing or not, showed a cumulative effect on absolute and relative intake of alcohol in the same pairs of high- and low-drinking lines. Consistent line differences in alcohol drinking behavior resulting from initial forced versus choice forms of alcohol exposure would indicate common gene $\times$ environment (epigenetic) interactions for all pairs of selected Indiana lines (see Chester et al., 2004; Chester et al., 2002; Crabbe et al., 1990).

To accurately monitor the rats' daily intake patterns of alcohol and water, our rats were housed and tested in individual experimental chambers isolated visually and by a white masking noise in a laboratory setting. We tested the importance of alcohol forcing on subsequent intake of alcohol and water by exposing age-matched groups within each pair of Indiana high- and low-drinking rat lines to an initial 4 days of either forced alcohol (Group F-C) or alcohol-water choice (Group C-C), both followed by a 22-day choice phase.

For each pair of high- and low-drinking lines, we determined: (1) alcohol intake during (a) the initial 4 days of exposure to either alcohol alone (Forced groups) or alcohol-water choice (Choice groups), and (b) the 22 subsequent days of alcohol-water choice; (2) the extent to which asymptotic alcohol and water intake during choice phase for the Forced and Choice groups met the intake criteria used in the School of Medicine colony to select rats for subsequent breeding; (3) whether intake scores met the criteria or not, could they be used empirically to differentiate rats in high- and low-drinking lines; and (4) whether imposing 4-days of forced alcohol on a subset of P and HAD2 Choice group rats, which showed a low-drinking phenotype during alcohol-water choice, would produce a high-drinking phenotype during a second choice phase.

Materials and Methods

Rats Tested in Laboratory Chambers

Subjects—Male rats: 16 P and 16 NP from generation 53, first exposed to alcohol between 101 and 111 days of age, weighing between 332 and 515 grams; 16 HAD1 and 16 LAD1 evenly

divided between generations 40 and 41, first exposed to alcohol between 98 and 151 days of age, weighing between 226 and 476 g; 16 HAD2 and 16 LAD2 from generation 37, first exposed to alcohol between 133 and 218 days of age, weighing between 286 and 459 g. The range of generations, ages, and weights of rats tested in our laboratory occurred because of logistics and design aims, including: matching generation, age, and weight for each of two replications of 16 rats in each pair of lines (4 in each combination of forced (F-C) versus choice (C-C) groups with high-drinking versus low-drinking lines), thoroughly acclimating the rats to the laboratory testing chambers before introducing alcohol, and initially ensuring the apparatus functioned without error.

One HAD2 rat and one HAD1 rat were subsequently removed from their C-C groups due to illness during the experiment, producing final total Ns of 15 HAD1s and 15 HAD2s. All experimental procedures were approved by the Bloomington Institutional Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Apparatus: Laboratory Experimental Chambers: During the experiment, each animal was housed individually in one of sixteen standard Med-Associates (St. Albans, VT) experimental chambers designed for adult rats (31 cm long × 24 cm deep × 21 cm tall). The experimental chambers were located in two metal cabinets (122 cm × 43 cm × 185 cm) with four shelves, two chambers per shelf. The cabinets were ventilated with a fan mounted on the top of the cabinets, and each shelf was illuminated on a 12:12 hr light-dark cycle by a 13-watt fluorescent light mounted above each shelf. There were two bottles on the left wall of each experimental chamber, one 3.5 cm from the front door and the other 3.5 cm from the back of the chamber. The two bottles were 17 cm apart and had sipper tubes with ball bearings to limit leaking. The tubes were 1.9 cm inset from the wall and photo-beam lick-o-meters (Med-Associates, VT) measured the licks on each bottle. In the center of the right wall was the feeder 6 cm × 2.5 cm × 5.7 cm. A photo-beam detected the presence of a 97-mg Noyes Formula P pellet (Research Diets; New Brunswick, NJ). When a pellet was removed from the feeder, the response was automatically recorded and another pellet was dispensed 1 second later. The floor of each chamber consisted of stainless metal bars, .48 cm in diameter and separated 1.6 cm on center.

Procedures

Acclimation and Daily Maintenance: To allow rats to acclimate to the novel chambers, water bottles, and food pellets, they were placed in the chambers 17–30 days prior to their first exposure to alcohol. Both drinking bottles contained distilled water. Lights came on at 07:00 and went off at 19:00. Each day between 14:30 and 15:30 there was a maintenance period when a familiar experimenter entered the room to weigh the rats and the bottles and refill the bottles and feeders, as appropriate. Daily water intake was measured by weighing each bottle and subtracting the result from its weight the previous day. Bottle positions were switched daily to control for the effects of any position preferences when alcohol was present. All sensors were checked and adjusted as necessary. Waste pans and chambers were cleaned once a week.

Initial Alcohol Exposure (4 days): At 14:30 on four successive days, a bottle of 10% alcohol (v/v with distilled water) was placed on each chamber. For the Choice group rats (C-C), a bottle of distilled water accompanied the alcohol bottle; for the Forced group rats (F-C) a single bottle of alcohol was the only liquid available. The position of the alcohol bottle was switched each day, along with the position of the water bottle (when present).

Choice Phase (22 days): All rats were provided one bottle of distilled water and one bottle of 10% alcohol. The positions of the alcohol and water bottle were switched daily.

Post-Choice Phase Alcohol Forcing in Selected Animals: Five *high-drinking line* rats (4 P rats and 1 HAD 2) in C–C groups (thus, never exposed to Forced alcohol) expressed a low drinking phenotype during their first choice phase. To further test the role of forcing, these 5 rats were subsequently forced with 4 days of alcohol alone, followed by a second choice phase of 20 days. Because of space limitations, these rats were moved from the experimental chambers to tubs with bedding and rat chow for this portion of testing. However, the light-dark cycle and the caretaking and measurement procedures remained the same, with daily weighing, measurement of intake, and switching of bottle positions between 14:30 –15:30.

Body weight and consumption of alcohol and water were measured in grams, once a day. To control for differences in body weight within groups and across lines, we standardized the grams of individual alcohol intake by dividing by the weight of each animal (g/kg). We averaged all measures across 2-day Blocks to eliminate any “saw-tooth” alcohol intake effect caused by a combination of subjects typically drinking more when alcohol was presented in the back location as compared to the front (nearest the door to the chamber), as well as the tendency for peaks of free intake of alcohol and water to oscillate by days (see Files et al., 1993). Averaging across 2-day Blocks also decreased the likelihood that the effects of changes between conditions would be biased by the bottle positions of the dominant fluid on the shift day. All analyses, unless otherwise noted, used repeated-measures Analysis of Variance (ANOVA), with post-hoc analyses using a Tukey’s HSD test. Also unless otherwise noted, all statistics reported are significant at $p < .05$.

Also, we compared alcohol intake of each high-drinking line to its minimum selection criteria of 5.0 g of alcohol/kg/day and a ratio of 2:1, alcohol to water (Li et al., 1993; McBride and Li, 1998; Murphy et al., 2002). Alcohol intake for the low-drinking lines was compared to its maximum standard of 1.5 g alcohol/kg/day and a ratio of 0.5:1, alcohol to water. Finally, because our testing circumstances were not the same as the colony (see below), we also asked whether the high- and low-drinking groups of each pair of lines tested and housed in our laboratory chambers differed in absolute intake measures (without regard to whether they met colony breeding criteria). Toward this end, we applied an empirical classification algorithm (a k-means cluster analysis) to the average intake measures of amount drunk and proportion of alcohol to total fluid. Each cluster analysis was driven by two goals: 1) minimizing variability within clusters and 2) maximizing variation between clusters (Statistica 6.0, Statsoft, Tulsa, OK).

Rats Tested in the School of Medicine Colony

To compare the drinking results for the rats tested in our experimental chambers with drinking results for rats tested in the School of Medicine breeding colony, we obtained the alcohol and water intake data and body weights from rats in generations similar to those in the lab, but were tested in the colony.

Subjects—Male rats: 48 P and 22 NP from generations 56 and 57; 27 HAD1 and 20 LAD1 from generation 43; and 21 HAD2 and 25 LAD2 from generation 38. The P/NP rats were within 4 generations of our laboratory-tested rats, the HAD/LAD1 rats were within 3 generations, and the HAD/LAD2 rats were within 1 generation. All colony rats received their first (forced) exposure to alcohol at approximately 60 days of age and were housed individually in hanging cages. Laboratory-tested rats varied in age of initial exposure, but were always older because of logistical requirements.

Procedures—In the colony procedure all animals received 4 initial days of forced alcohol followed by 21 days of choice. During the choice phase, each rats had ad lib access to two Richter tubes (providing large fluid surfaces to lap from); one contained water, and the other

contained a 10% (v/v) alcohol solution. Total intake of water and alcohol were measured twice a week and the bottle positions were switched during each reading. The final measure for each rat on the last day of choice constituted the test data presented here. Rats had ad lib access to commercial rat chow, and were weighed once per week. The colony-housed rats had less visual and auditory isolation than did the laboratory rats in their experimental chambers, and the bottles were switched less frequently. All rats lived under a 12:12 light:dark cycle.

Results

In this section we display and analyze the results of the Forced and Choice Groups of each pair of high and low-drinking lines in four parts: (1) alcohol intake during (a) the initial 4 days of choice or forced alcohol, and (b) the 22 days of the alcohol-water choice phase; (2) the extent to which laboratory-tested Forced and Choice groups for each pair of high- and low-drinking lines satisfied the colony selection criteria by the end of 22 days of alcohol-water choice; (3) the extent to which an empirical multi-variate classification technique distinguish phenotypes of high- and low-alcohol-drinking animals within a particular pair of lines; and (4) the effect of subsequently forcing alcohol on a subset of genetically high-drinking Choice group rats that expressed a low-drinking phenotype during the 22 day choice phase.

Alcohol Intake by Laboratory-Tested Forced and Choice Groups

Figure 1 shows intake of alcohol for Forced and Choice groups of the three pairs of alcohol preferring and non-preferring lines (P-NP; HAD1-LAD1; HAD2-LAD2). The data are scaled in g/kg of body weight and plotted in thirteen 2-day Blocks across the initial alcohol exposure phase (Forced or Choice, Blocks 1–2) and the subsequent choice phase (Blocks 3–13).

P-NP Lines (top figure)

(1). Initial Exposure: During initial exposure to alcohol (Forced or Choice), an ANOVA showed that P rats drank more alcohol than NPs, F-C (Forced) rats drank more alcohol than C-C (Choice) rats, and more alcohol was consumed on the second two-Day-Block than on the first [$F_s(1,28) = 8.8, 83.9, \text{ and } 5.5$, respectively]. There also was a significant interaction of 2-day Blocks $\times$ Group [$F(1, 28) = 15.0$]. Tukey's tests showed: (a) F-C rats increased alcohol intake on the second forced alcohol Block, but C-C rats did not. (b) Both P and NP F-C groups drank more alcohol than P and NP C-C groups. (c) There were no differences in alcohol intake between P C-C and NP C-C rats, or between P F-C and NP F-C rats; although the difference between the latter two groups approached significance ($p = 0.062$).

(2). Transition: During the transition to the choice phase (last Block of initial alcohol exposure and first Block of choice), P rats drank more than NP rats, F-C rats drank more than C-C rats, and less alcohol was consumed in choice phase than in the initial alcohol phase [$F_s(1,28) = 12.4, 30.0, \text{ and } 100.4$]. There were also significant interactions of Line $\times$ Phase, Group $\times$ Phase, and a three-way interaction of Line $\times$ Group $\times$ Phase [$F_s(1,28) = 9.1, 144.2, \text{ and } 11.3$, respectively]. Tukey's tests showed that only the F-C groups (P and NP) decreased alcohol consumption.

(3). Choice Phase: During the choice phase, P rats drank more than NPs [$F(1,28) = 36.1$], alcohol intake increased across 2-day Blocks, and there was an interaction of 2-day Block $\times$ Line [$F_s(10,280) = 2.7 \text{ and } 2.5$, respectively]. Looking at individual groups, P C-C rats increased alcohol intake across choice phase [$F(10,70) = 3.1$]; NP C-C rats decreased alcohol intake after the first Block [$F(10,70) = 2.0$]; P F-C and C-C groups did not differ significantly in alcohol intake at either the beginning or end of the choice phase, although the beginning approached significance [$F(1,14) = 4.1, p = 0.063$].

HAD1-LAD1 Lines (middle figure)

(1) Initial Exposure: During initial alcohol exposure, HAD1 rats drank more alcohol than LAD1s, and F-C rats drank more than C-C rats [$F(1,27) = 26.1$ and 91.0 , respectively]. A Tukey's test showed that HAD1 F-C rats drank significantly more alcohol than all other groups and that LAD1 F-C rats drank more than either the LAD1 C-C or HAD1 C-C groups.

(2) Transition: During the transition to choice phase, HAD1 rats drank more than LAD1s, F-C rats drank more C-C rats, more alcohol was drunk overall on the last 2-day Block of initial alcohol than the first 2-day Block of choice phase, and there was a significant interaction of Phase $\times$ Group [$F_s(1,27) = 30.9, 27.5, 92.4$, and 29.9 , respectively]. Tukey's tests showed that F-C rats drank more alcohol than the C-C groups in both phases, and that both HAD1 F-C and LAD1 F-C rats decreased alcohol intake significantly at the onset of the choice phase.

(3) Choice Phase: During the choice phase, HAD1 rats drank more than LAD1s [$F(1,27) = 62.4$], there was an overall increase in alcohol intake across Blocks, and there was a Line $\times$ 2-day Block interaction [$F_s(10,270) = 4.8$, and 6.0 , respectively]. Tukey's tests showed that significantly less alcohol was consumed at the beginning of the choice phase (Blocks 3 and 4) than at the end (Blocks 12 and 13), and that both HAD1 F-C and C-C groups drank more than LAD1 F-C and C-C rats. The only group to show a significant increase of alcohol consumption across the choice phase was the HAD1 C-C rats [$F(10,60) = 3.6$]. Despite decreasing alcohol intake following the Forced Phase, the HAD1 F-C rats drank more alcohol than HAD1 C-C on the first Block of the choice phase [$F(1,13) = 5.2$]. However, after the first choice Block, alcohol intake did not differ between HAD1 F-C and HAD1 C-C groups. Even at the end of the choice condition, the HAD1 F-C rats drank less alcohol than at the end of the Forced Phase [$F(1,7) = 5.6$].

HAD2-LAD2 Lines (bottom figure)

(1) Initial Exposure: During initial exposure to alcohol, HAD2 rats drank more alcohol than LAD2s, F-C rats drank more than C-C rats, and more alcohol was consumed on the second 2-day Block than on the first [$F_s(1,27) = 15.9, 38.6$, and 13.5 , respectively]. A Tukey's test showed that HAD2 F-C rats drank more alcohol than all other groups. LAD2 F-C rats drank more alcohol than the LAD2 C-C rats, but alcohol intake for HAD2 C-C rats was intermediate and only greater than the LAD2 C-C group.

(2) Transition: During the transition from initial alcohol exposure to choice, HAD2 rats drank more alcohol than LAD2s, F-C rats drank more than C-C rats, and more alcohol was consumed on the last initial alcohol Block than on the first Block of choice phase [$F_s(1,27) = 23.0, 29.8$, and 19.2 , respectively]. Furthermore there was a 3-way interaction of Line $\times$ Group $\times$ Phase [$F(1,27) = 10.3$]. A Tukey's test showed that only the LAD2 F-C group significantly decreased their alcohol consumption from forced to choice, all other groups were unchanged.

(3) Choice Phase: During the choice phase, HAD2 rats drank more alcohol than LAD2s [$F(1,27) = 36.8$] and there was an overall increase in alcohol intake across Blocks [$F(10,270) = 5.6$]. A Tukey's test showed that Blocks 10 through 13 were each greater than Blocks 3 and 5. There were also interactions of Line $\times$ 2-day Block and Group $\times$ 2-day Block [$F_s(10,270) = 5.6$ and 2.5 , respectively]. Looking at individual groups, only HAD2 C-C rats increased alcohol intake across Blocks of choice [$F(10,60) = 6.1$]. A Tukey's test shows that alcohol drinking on Blocks 8 through 13 was significantly higher than on the first Block of choice, Block 3. Thus, the HAD2 C-C group accounts for the bulk of the overall effect of increased alcohol intake across Blocks. The HAD2 C-C rats started out drinking significantly less alcohol than HAD2 F-C rats [$F(1,13) = 6.0$], but increased their intake across Blocks to a level statistically indistinguishable from the F-C rats by the end of the choice phase. The LAD2 groups also

showed significant variation in alcohol intake across choice Blocks, but the F-C rats showed no consistent direction, and the C-C group showed only a small increase on the last day of the choice phase.

Did Laboratory-Tested, High- and Low-Drinking Lines Meet Colony Selection Criteria?

Figure 2 shows the extent to which laboratory-tested pairs of high- and low-drinking lines satisfied the colony selection criteria for absolute and proportional alcohol intake, based on the average of the last four days of the 22-day choice phase. Each graph shows the scores of individuals from one pair of high- and low-drinking lines (P-NP rats in the top graph, HAD-LAD1s in the middle, and HAD-LAD2s on the bottom). Each figure shows *low-drinking line* rats as open and filled circles and *high-drinking line* rats as open and filled triangles. The open symbols correspond to Choice group rats and the filled symbols to Forced group rats.

Each symbol designates the average grams of alcohol intake/body-weight (seen on the y axis) and the average percent alcohol ml to total fluid ml (seen on the x axis) for that individual. Thus, the symbols for rats meeting both colony alcohol selection criteria for low drinking are found in the box at the lower left corner of each figure, while the symbols for rats meeting both colony alcohol selection criteria for high drinking are found in the box in the upper right corner of each figure. The locations of the symbols of the remaining rats show the extent to which an animal met one, or the other, or neither breeding criterion for its line.

P-NP Rats—The top graph of Figure 2 shows the results for individual rats in the P-NP pair of high and low-drinking lines. All 16 NP rats, regardless of whether they were in the Forced or Choice group in their initial alcohol exposure, are found in the lower left box in the figure, showing that they met both breeding criteria for low-drinking lines. In marked contrast, only a single P F-C rat met both the high-drinking selection criteria (see the upper right box). Three of the 16 P rats passed the 2:1 alcohol-to-water criterion alone, while the remaining 12 P rats failed to satisfy either selection criterion.

HAD1-LAD1 Rats—The middle graph of Figure 2 shows the results for individual rats in the HAD1-LAD1 pair of high- and low-drinking lines. Again, all 16 low-drinking rats (LAD1s), whether in the Forced or Choice group for initial alcohol exposure, met both breeding criteria (see the lower left box). In contrast, only three Choice rats out of the 15 HAD1 rats met both breeding criteria (see the upper right box). Five more HAD1 rats met the 2:1 alcohol-to-water criterion, 1 met the 5 g/kg alcohol criterion, and 6 rats met neither criterion.

HAD2-LAD2 Rats—The bottom graph of Figure 2 shows the results for individual rats in the HAD2-LAD2 pair of high- and low-drinking lines. In this case, 15 of the 16 low-drinking rats (LAD2s) clearly met both breeding. The one failure was a highly deviant forced LAD2 who drank amounts of alcohol and water very similar to rats of the high-drinking line. The HAD2 line came closest of all the high-drinking lines to meeting the high-drinking criteria, with 6 out of 8 of the Forced group and 3 out of 7 of the Choice group meeting both high-drinking criteria, while another two of the Choice group high-drinking rats passed the 2:1 alcohol-to-water criterion.

Given a minimum of 36 breeding generations for each pair of low- and high-drinking Indiana lines had met the colony selection criteria, it was not surprising that 47 of our 48 laboratory-tested, *low-drinking line* animals met both the absolute and relative low alcohol drinking criteria (as shown by the cluster of symbols in the lower left boxes in Figure 2). This effect occurred whether or not the rats were forced on alcohol prior to testing, suggesting that forcing with alcohol had little, if any, effect on the phenotypes of *low-drinking line* rats. However, it was unexpected that only 13 of the 46 *high-drinking line* animals (28.3%) tested in the

laboratory met both the colony criteria for absolute and relative high-drinking alcohol intake. Further, 9 of these 13 rats were in the HAD2 line, and the majority of those were in the Forced group.

In the next several paragraphs we consider three possible contributors to the unexpectedly infrequent expression of a high-drinking phenotype, particularly in the HAD1 and P lines: (1) there exists a high level of variability in expression of the high-alcohol drinking phenotype in each generation, requiring a high level of selection to choose the breeders of each new generation; (2) the initial 4-day forced exposure to alcohol prior to alcohol-water choice has become a critical contributor to the expression of a high-drinking phenotype; and (3) *high-drinking line* rats are more sensitive than *low-drinking line* rats to environmental and procedural components in our laboratory that differed from those present in the School of Medicine colony.

Figure 3, plotted using the same axes, criteria boxes, and symbols as Figure 2, allows us to compare easily absolute and proportional alcohol drinking during alcohol-water choice for high-drinking and low-drinking lines tested in our laboratory chambers versus high-drinking lines tested in the colony. As in Figure 2, P-NP rats are shown in the top graph, HAD1-LAD1 rats in the middle graph, and HAD2-LAD2 rats in the bottom graph. Also as in Figure 2, the triangles represent intake data from individual *low-drinking line* rats, and the circles represent individuals from high-drinking lines. All symbols are filled in Figure 3 to indicate that all rats were forced in the colony procedure. The colony procedure was very similar to the laboratory procedure in that each animal received 4 initial days of forced alcohol followed by 21 days of choice (22 in the laboratory). Intake was measured only twice a week (as opposed to each day in the laboratory) and the final measure for each rat constituted the test data. We were interested primarily in the relative variability in drinking scores of the high-drinking rats.

It can be seen by comparing Figures 2 and 3 that the colony- and laboratory-tested, *low-drinking line* rats (triangles) were very similar in their alcohol ingestion. A minor difference was that the colony-tested LAD1s and LAD2s were slightly more tightly clumped near zero on both criterion measures. However, comparing the scores of the *high-drinking line* rats in Figures 2 and 3 showed a larger dispersion in the drinking scores, particularly for the laboratory-tested animals, which generally failed to meet the colony selection criteria and were noticeably lower on both absolute and proportional measures of alcohol intake. Each colony-tested, high-drinking line met both criteria at least 87% of the time.

In sum, the vast majority of the rats in low-drinking lines showed a high degree of similarity in alcohol and water intake, whether tested in the School of Medicine colony or in our laboratory chambers (and in the latter case, whether forced or not forced with alcohol prior to the choice test). These data support the argument that laboratory and colony test conditions were equivalent in their influence on alcohol and water intake for the low-drinking lines. In contrast, alcohol and water intake in the high-drinking lines tested in our laboratory showed notably greater individual variability and failure to meet selection criteria than the same lines tested in the colony.

Finally, several factors may have contributed to the differences in alcohol intake between laboratory-tested and colony-tested, high-drinking rats. First, the laboratory-tested rats were 98 or more days of age when initially exposed to alcohol, while the colony-tested rats were exposed to alcohol at 60 days of age. Older rats are also typically heavier rats. Table 1 explores the possible importance of age and weight by showing the outcome of Pearson product-moment correlations calculated between alcohol intake, averaged across the last 4 days of the choice phase, and age (columns 1 & 2), or weight (Columns 3 & 4). The correlations were computed separately for the Forced and Choice groups for each high-drinking line. We also computed

overall correlations by combining animals within each line and across all three high-drinking lines.

None of the correlations were statistically significant, and there was no consistent relation between alcohol intake and either age or weight within each of the three high-drinking lines, or when all were tested together. Further, although HAD1 alcohol intake tended to be negatively related to both age and weight, HAD2 intake was slightly negatively related to age, and slightly positively correlated with weight, while alcohol intake measures for P rats showed even less relation to either age or weight. Based on these data, any effect of age or weight on alcohol intake would appear to be minimal and inconsistent.

Empirically Distinguishing High and Low Drinking Rats in the Laboratory

Despite the markedly lower alcohol intake in the high-drinking lines tested in laboratory chambers relative to the intake of similar generations of these lines tested in the colony, inspection of the data in Table 2 clearly shows that over the last four days of the choice phase the laboratory-tested, *high-drinking line* rats still averaged greater absolute and proportional alcohol intake than the *low-drinking line* rats. This held for both Forced and Choice groups and across all three pairs of Indiana lines. The left two columns show group means for each selection criterion and the proportion of rats that met each criterion.

To determine if the visible differences between high- and low-drinking lines on both alcohol drinking criteria were reliable, we subjected the data to an empirical multi-variate classification technique (k-means cluster analysis, STATISTICA 6, StatSoft, Inc. 2004). When applied to the average alcohol and water intake over the last four days of each choice phase, this test allowed us to simultaneously use both alcohol intake measures (absolute and proportional) to empirically divide rats into high- and low-drinking groups for each pair of lines (P-NP, HAD1-LAD1, HAD2-LAD2). In addition to the ability of k-means clustering to consider multiple measures at the same time, this method also imposes no a priori decision rule for how many subjects should be in each group. As a result membership in the empirically differentiated high- and low-drinking groups is constrained only by a computational algorithm that simultaneously maximizes the variance between resultant groups and minimizes the variance within them.

The results (see the third column of Table 2) show the proportion of animals in each genetic group that were correctly identified as *high-* or *low-drinking line* animals by k-means clustering based on both measures of alcohol intake. This analysis showed noticeably better agreement with the genetic history of the lines than did comparison of our rats with the selection criteria of the breeding colony. In the Forced groups the cluster analysis correctly classified, according to their genetic line, 6 out of 8 of the P rats, 6 out of 8 of the HAD1 rats, and 7 out of 8 of the HAD2 rats. In the Choice groups the cluster analyses correctly classified 5 out of 8 of the P rats, 5 out of 7 of the HAD1 rats, and 5 out of 7 of the HAD2 rats. Applying the same procedures to *low-drinking line* rats resulted in only a single misclassified animal, a LAD2.

Effects of Forcing Alcohol on *High-Drinking Line* Choice Rats Classified as Low Drinkers—Our final concern was to evaluate the contribution of the colony procedure of forcing all animals with four days of alcohol alone prior to their three week choice phase. Although absence of forcing alcohol intake cannot alone account for the failure of our *high-drinking line* animals to reach the colony selection criteria for breeding, close inspection of Table 2 and Figure 2 shows that noticeably more *high-drinking line* rats from Choice groups than from Forced groups drank low amounts of alcohol and were empirically classified as low-drinkers.

This difference between Choice and Forced groups in producing high-drinking alcohol phenotypes suggests that forcing may be an important determinant of subsequent choice phase

alcohol intake by *high-drinking line* rats in the laboratory. To test this hypothesis, and cast some light on whether forcing alcohol can affect intake after (as well as before) a choice phase, we forced alcohol on the four lowest drinking rats in the P Choice group for 4 days followed by a second 20 days of choice. We used the same procedure with a single low-drinking HAD2 rat. Unfortunately, by the time we thought of testing the effects of post-choice forcing, we had finished running the HAD1 Choice rats and half of the HAD2s, so we were not able to use this test procedure on low-drinking rats from these groups.

Figure 4 shows the amount of alcohol drunk by the five C-C rats during the first 13 Blocks of alcohol-water choice (2 Blocks of initial exposure and 11 of choice phase), 2 Blocks of forced alcohol, and 10 Blocks of a second choice phase. All five of these low-drinking C-C rats from high-drinking lines markedly increased their alcohol intake during the second choice phase, meeting the 2:1 alcohol to water criterion. Repeating the k-means cluster analysis for all P and NP rats now assigned all four of the previously low drinking rats to the high drinking group. The tested low-drinking HAD2 Choice rat showed a remarkable increase in alcohol intake after four days of forced alcohol. At the end of the subsequent choice phase, it consumed over 8.9 g of alcohol per kg bw per day, with a ratio of 6.9:1, alcohol to water, meeting both colony criteria for high-drinking breeders, and was empirically classified as a high-drinker by a k-means cluster analysis. Visual comparison of the second part of Figure 4 to Figure 1 shows that the intake of these animals is similar to that of rats from the same strains that were initially forced.

Discussion

Here we consider: (1) common patterns of how alcohol intake among the three pairs of laboratory-tested, low- and high-drinking lines relate to circumstances of alcohol and water availability (forced or choice), (2) the role of selection and testing procedures in producing a high-drinking phenotype, and (3) the indication that the form of alcohol exposure influences the expression of a high-drinking, but not a low-drinking, phenotype.

Common Patterns of Alcohol Intake

Alcohol intake during the first four days of alcohol exposure in all three pairs of laboratory-tested lines was higher for the Forced groups than for their related Choice groups. Alcohol intake was higher for the high-drinking lines than for their low-drinking counterparts. The difference in alcohol intake between the high- and low-drinking lines was smallest for P/NP lines and highest for the HAD2/LAD2 lines, primarily because Forced P rats drank relatively less than Forced HAD2 rats. Samson et al. (1998) also found that P rats had significantly lower alcohol intake in their home cages than HAD1 or HAD2 rats. However, this was true only for the g/kg measure and not for the measure of percent alcohol in total fluid intake. In the current study laboratory-tested P and HAD rats showed a similar pattern of differences.

During the 22-day choice phase, alcohol drinking by the Forced and Choice groups converged for both high-drinking and low-drinking line animals, although in different ways. For low-drinking lines, alcohol intake of the Forced group dropped immediately to the level of related Choice controls on Block 1 of the choice phase and remained at that level for the full 22 days. For high-drinking lines, the alcohol intake on the first 2-day Block of the choice phase for Forced groups was higher than that of Choice groups never forced. However, the picture is more complex in two respects. First, the majority of animals in a Choice group slowly increased their intake of alcohol over the 22 days of the choice condition to a point not significantly different from the scores of the Forced group. Second, a subset of the *high-drinking line* Choice group animals never showed an increase in alcohol intake over the choice phase. We will return to this result shortly.

Effects of Testing Circumstances and Procedures on Alcohol Intake

Animals in laboratory-tested, low-drinking lines showed stable drinking phenotypes similar to those reported for colony-tested, low-drinking lines. Only one of 48 *low-drinking line* animals exceeded the limiting criteria of 1.5 g/kg/day of alcohol and a 0.5:1 ratio of alcohol to water during the choice phase. In contrast, well over half of our laboratory-tested Ps and HAD1s (and nearly half of the HAD2s) failed to reach the high-drinking selection criteria of 5.0 g/kg/day of alcohol and a 2:1 ratio of alcohol to water by the end of the choice phase. However, the intake differences between laboratory-tested, high- and low-drinking animals in each pair of lines were still present. An empirically-grounded k-means cluster analysis, based on maximizing variance between “candidate” groups while minimizing variance within them, revealed stable and distinct high- and low-drinking phenotypes in each pair of lines.

The reliable expression of separate high- and low-drinking phenotypes in the Choice groups means that forcing is not required in most cases to produce different drinking phenotypes. For most *high-drinking line* choice animals, all that is required to systematically increase alcohol intake to approximately the level resulting from forcing is an extended (2–3 week) exposure to alcohol-water choice. However, two further points can be made. First, for most *high-drinking line* animals, forcing appears to increase the speed with which the high-drinking phenotype is expressed. Second, there appears to be a subset of *high-drinking line* animals that do not increase alcohol intake under alcohol-water choice; instead they require forcing to produce a high-drinking phenotype.

This leaves us with the final question of why the *high-drinking line* phenotype expressed by laboratory-tested rats receiving only a choice phase showed such a markedly low absolute and proportional intake of alcohol relative to the standard selection criteria. Consider that at least 87% of colony-tested, *high-drinking line* rats by the end of the choice phase satisfied both alcohol-drinking criteria, while few laboratory-tested, *high-drinking line* rats satisfied even one. Several possible causes should be raised. First, laboratory rats were older than colony rats when we tested them under alcohol-water choice. Age may directly reduce alcohol intake, perhaps by producing increased sensitivity to the disruptive effects of alcohol on gross motor performance (see Draski et al., 2001). Further, because alcohol is more soluble in water than fat, older rats may be affected by a smaller dose of alcohol because they have proportionally more fat relative to overall body mass (see Chester et al., 2003; Iossa et al., 1999; Masoro, 1980). In fact, the hypothesis that rats in high-drinking lines tend to take in an effective dose of alcohol could help explain why the P line (our heaviest, rather than our oldest animals), were the least likely to reach the alcohol ingestion criteria for high drinking lines.

Nevertheless, despite a wide range of ages and weights among our laboratory subjects, significant and consistent correlations between alcohol intake measures and age or weight were not shown in our laboratory tests (see Table 1). Given the absence of a significant contribution of age and weight to alcohol intake in our laboratory-tested, *high-drinking line* rats, and the fact that *low-drinking line* rats showed the same alcohol intake in laboratory and colony, it seems likely that some aspect(s) of the laboratory environment and testing procedures reduced alcohol ingestion in our laboratory-tested, *high-drinking line* animals relative to their colony-tested counterparts.

With respect to physical conditions, both laboratory and colony circumstances used a 12:12 light:dark cycle; however, laboratory experimental chambers had more visual and auditory isolation than did individual colony cages. The laboratory rats were fed Noyes pellets instead of blocks of chow and obtained alcohol by licking from bottles with sipper tubes rather than the colony Richter tubes, the latter having a large surface to lap fluid from. In the absence of additional obvious differences, it seems worth pointing out that the laboratory rats were handled daily (to take weights), and that the positions of alcohol and water bottles in the apparatus were

switched each day. Regular handling has been shown to reduce distress (Holson, 1986) and switching the position of the water and alcohol bottles would require each rat to relearn their position on a daily basis, thereby possibly resulting in less difference between intake of alcohol and water solutions.

The Importance of Forcing Alcohol in Laboratory-Tested Rats

As noted above, the usual procedure for selecting high- and low-drinking lines involves forcing animals on alcohol as their only fluid for 4 days prior to testing choice between alcohol and water for a 3-week period. When laboratory-tested, *high-drinking line* animals were not forced during their first 4 days of exposure to alcohol, but simply exposed to a choice of alcohol and water, they generally took in significantly less alcohol on the first Block of the choice phase than did their forced counterparts. Forcing alcohol rapidly increased the preference for alcohol ingestion in the three high-drinking lines relative to that expressed by animals in the same lines exposed only to the choice condition.

High-drinking line—Choice groups showed an increase in average ingestion of alcohol within 6 to 10 days during the alcohol-water choice phase. This relatively slow, steady increase suggests the possibility of a mildly positive feedback mechanism using each increase in alcohol intake to set the stage for a further increase, a mechanism that seems to eventually approach a point of alcohol intake similar to that produced by forcing. It may be that alcohol consumption activates gene-based regulatory and metabolic mechanisms important for the maintenance of high alcohol drinking behavior (or perhaps results in permanent changes in gene expression through epigenetic mechanisms that maintain high alcohol drinking behavior). It is worth noting that reprogramming of gene expression has been found in long-term human alcoholics (Mayfield et al., 2002). In contrast, four days of forced alcohol appears to result in a high dose of alcohol which accelerates these effects so that high-drinking appears on the first or second day of alcohol-water choice.

The presence of a subset of *high-drinking line* Choice animals that showed little alcohol intake over 26 days of choice exposure, but increased intake immediately after being forced, argues that some animals require forcing to express a high-drinking phenotype. As shown in Figure 4, once these phenotypically low-drinking P and HAD2 rats were forced they maintained a high-level of alcohol intake throughout the second choice condition. It is unfortunate that we did not appreciate the potential information provided by this secondary (post-choice) forcing until after the HAD1 rats finished testing. Their results would have provided additional data on the extent to which independently selected lines of high- and low-alcohol-drinking rats converge on a high-drinking phenotype initiated by forcing.

In short, the standard procedure of forcing animals to drink alcohol prior to alcohol-water choice appears to have made an important contribution to the selection of genotypically diverse high-drinking rat lines. Our laboratory data suggest multiple pathways for expression of the high-drinking genotype, each depending on the form of alcohol experience and the testing circumstances. One path involves forced exposure to alcohol, although the effects of alcohol concentration and duration of forcing need to be clarified. A second path involves repeated exposure to a choice between alcohol and water. Most *high-drinking line* animals exposed only to an alcohol-water choice condition showed a noticeable increase in alcohol intake within 6–10 days, though with variation across individuals and genetic stock. For example, on average HAD1 rats increased alcohol intake more rapidly, P rats more slowly, and individuals within each line varied in the rate of increase. A third path involves animals that showed little alcohol intake under choice conditions until they were forced, though this may involve mechanisms similar to initial forcing. The extent to which these exposure pathways are based on expression of similar genetic alleles is not yet clear. Additionally, in the initial forced exposure path, the

extent to which the expression of the high-drinking phenotype depends on the environment and procedures (laboratory vs. colony) requires clarification.

It seems to us that the exposure-related effects produced by manipulating alcohol availability, rather than indicating a potential problem in using *high-drinking line* animals as a model of human alcohol intake, may speak to a genetic complexity in these rat lines that parallels a similar complexity in human populations. For example, rats that respond to long term exposure to alcohol-water choice may serve as a model of human alcoholics who gradually increase their intake of alcohol across choice conditions. On the other hand, genetically *high-drinking line* animals that require forcing to produce a preference for alcohol under choice conditions, might serve as a model of potential human alcoholics who react to bingeing by subsequently increasing their intake of alcohol under choice (Jennison, 2004).

It would be interesting to know if these *high-drinking line* subgroups showed consistent similarities and differences in other measures of alcohol ingestion, such as operant self-administration (Czachowski and Samson, 2002) or reaction to sweetened alcohol solutions. For example, exposure to sweetened alcohol solutions under free-choice conditions has been shown to significantly increase alcohol drinking in NP rats to a comparable level to that seen in P rats (Gauvin et al., 1998; Waller et al., 1982). However, Tampier and Quintanilla (2005) have shown that prior exposure to sweetened alcohol enhances unsweetened alcohol consumption in rats selectively bred for high alcohol drinking (UChB) but not in those selectively bred for low alcohol drinking (UChA). Sweetened alcohol solutions might be used to determine if there is a threshold of alcohol intake (comparable to being forced) necessary to produce a high-drinking phenotype in subsequent choice conditions for the Indiana lines.

Finally, in the case of the *low-drinking line* animals, the absence of a forced-alcohol effect, and the lack of differences between the alcohol intake of colony- vs. laboratory-tested, *low-drinking line* rats, suggests that their preferences are not affected by exposure procedures and differences between colony and laboratory testing environments. One possible explanation is that the amount of alcohol ingested is so small that the detrimental effects of intake based upon the type of exposure to alcohol (or testing location) is not accurately assessed by daily intake in the choice paradigm. Another possibility is that the alcohol intake of low-drinking rats is more directly dependent on taste and smell stimuli, rather than solely by effects of alcohol (Gauvin et al., 1998). Manipulations such as boosting alcohol's attractiveness and testing forced vs. choice subjects in conditions more similar to the breeding colony appear to be important areas for additional work.

Summary

In all three pairs of laboratory-tested lines, the high-drinking line averaged greater alcohol intake than the low-drinking line, and there were similarities among the three high-drinking lines and among the three low-drinking lines. In addition, each pair of lines showed characteristic empirical differences between high- and low-drinking animals in terms of alcohol ingested. Most importantly, it appears that the expression of a high-drinking phenotype was influenced by the path of prior exposure to alcohol. Without forcing, some *high-drinking line* animals failed to show a high-drinking phenotype, although most increased alcohol intake across a period of alcohol-water choice. Forcing alcohol in the high-drinking lines, either initially or after alcohol-water choice, increased alcohol intake to a high level with few exceptions. That independently selected high-drinking lines can show both a choice and a forced form of high-alcohol drinking suggests overlap in the actions of underlying genes selected for in different lines.

In terms of modeling alcohol intake in humans, further study of the subgroups of *high-drinking line* animals might contribute to understanding alternate paths to high levels of alcohol intake

in humans. Most *high-drinking line* Choice animals required only continued exposure to alcohol-water choice to produce a preference for alcohol, but some showed a high-drinking phenotype only after 4 days with alcohol as the only fluid. It seems worth exploring the potential similarity of these latter rats to the population of humans who begin to increase intake after binge-drinking episodes (Jennison, 2004). Finally, at a practical level, to establish the most consistent voluntary drinking phenotype for Indiana rat lines, investigators should ensure that all *high-drinking line* animals receive forced alcohol exposure prior to the experiment.

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Figure 1.

Mean ($\pm$ SEM) Intake of alcohol in g/kg of body weight for P & NP (top), HAD1 & LAD1 (middle), and HAD2 & LAD2 (bottom) groups, plotted in 2-day Blocks across the four days (2 Blocks) of Initial Exposure to Alcohol (Forced or Choice Groups) and the 22 days (11 Blocks) of the choice phase. Rats were housed individually in experimental chambers following adaptation to the chambers with only water and food available.

Figure 2.

Mean individual alcohol intake (g/kg/bw) and mean percent alcohol of total fluid intake for each laboratory-tested rat averaged across the last four days of the alcohol-water choice phase for P & NP groups (generation 53) (top), HAD1 & LAD1 groups (generations 40 & 41) (middle), and HAD2 & LAD2 groups (generation 37) (bottom). The boxes in the upper right and lower left represent the standard breeding selection criteria for the high- and low-drinking lines. Filled symbols denote the Forced group rats, which received four days of forced alcohol; the open symbols denote the Choice group rats, which were forced.

Figure 3.

Individual alcohol intake (g/kg/bw) and percent alcohol of total fluid intake averaged across the last four days of the choice phase for *high-* and *low-drinking line* colony rats from similar generations to the rats examined in Figures 1 and 2 above: P & NP generations 56 & 57 (top), HAD1 & LAD1 generation 43 (middle), and HAD2 & LAD2 generation 38 (bottom). The boxes in the upper right and lower left of the figure show the colony breeding selection criteria for the high- and low-drinking lines. All rats received four days of forced alcohol prior to choice testing. These data were recorded from rats housed in individual colony cages by colony laboratory technicians as part of the process of selecting breeders for the Indianapolis colony. The data were provided by Sherri Guthrie (IUPUI).

Figure 4.

Individual alcohol intake (g/kg/bw) by 2-day Blocks for 5 *high-drinking line* Choice (C-C) rats run in experimental chambers, during: (a) 13 Blocks of exposure to alcohol and water choice (2 Blocks of initial exposure, plus 11 Blocks of continued exposure), (b) 2 Blocks of exposure to alcohol alone (post-experiment forcing of alcohol), and (c) 10 Blocks of re-exposure to choice between alcohol and water. Four of the rats came from the P high-drinking line (P C-C group), and 1 rat from the HAD2 high-drinking line (HAD2 C-C group). The rat numbers correspond to the number assigned the rat at the beginning of the experiment. These particular rats were chosen for forcing because in choice phase they showed low levels of alcohol intake typical of their *low-drinking line* counterparts.

Table 1

Mean (Standard Error) and Person-Moment Correlation (r) of Age (Data Columns 1 & 2) and Weight (Data Columns 3 & 4) with Alcohol Intake (g/kg) for the Last 4 Days of Choice Phase for Laboratory-tested, High-drinking Lines.

Group	Age (Days)		Weight (g)	
	Mean ($\pm$ S.E.M.)	Mean Correlation Coefficient (r)	Mean ($\pm$ S.E.M.)	Mean Correlation Coefficient (r)
P F-C	106 (1)	-0.18	467 (16)	0.43
P C-C	105 (1)	0.37	515 (18)	-0.07
All P	105 (1)	0.04	491 (13)	-0.02
HAD1 F-C	123 (14)	-0.49	308 (14)	-0.34
HAD1 C-C	123 (7)	-0.50	300 (11)	-0.59
All HAD1	123 (5)	-0.46	304 (9)	-0.43
HAD2 F-C	181 (13)	-0.22	356 (21)	0.33
HAD2 C-C	178 (13)	-0.30	337 (13)	0.37
All HAD2	179 (9)	-0.22	345 (13)	0.38
All High-Drinking Lines	135 (6)	0.20	382 (14)	-0.27

Table 2
The Mean (Standard Error) and Proportion of Laboratory-tested, Genetically High- and Low-drinking Rat Lines Correctly Identified Based on Breeding Colony Alcohol Preference Criteria (Data Columns 1 & 2) and Proportion of Animals Correctly Identified by k-means Cluster Analysis (Data Column 3).

Group	Criterion 1 (5 g/kg/day)		Prop.	Criterion 2 (2:1 Ratio Alcohol:Water)			K-Means Cluster Classification Proportion
	Mean (S.E.M.)			Mean	Prop.		
P F-C	3.4 (.6)		1/8	1.9:1	3/8		6/8
P C-C	2.2 (.4)		0/8	1.1:1	1/8		5/8
NP F-C	0.8 (.08)		8/8	0.2:1	8/8		8/8
NP C-C	0.9 (.08)		8/8	0.2:1	8/8		8/8
HAD1 F-C	3.7 (.3)		1/8	1.9:1	3/8		6/8
HAD C-C	4.1 (.9)		3/7	2.8:1	5/7		5/7
LAD1 F-C	0.6 (.08)		8/8	0.1:1	8/8		8/8
LAD C-C	0.6 (.08)		8/8	0.1:1	8/8		8/8
HAD2 F-C	5.4 (.7)		6/8	10.8:1	6/8		7/8
HAD2 C-C	3.9 (.9)		3/7	6.0:1	5/7		5/7
LAD2 F-C	1.1 (.3)		7/8	1.8:1	7/8		7/8
LAD2 C-C	0.7 (.08)		8/8	0.2:1	8/8		8/8