STRESS INDUCED EVOLUTION OF NON-GENOTYPIC HETEROGENEITY

MRes Evolutionary Biology

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ABSTRACT

Genetically identical populations can exhibit a range of behaviours within a phenotype, despite sharing a genetic background. This is referred to as non-genotypic heterogeneity (NGH). NGH can be conferred by a heritable change in DNA that affects the noise surrounding expression of a protein product. Copper and zinc are trace metals that are essential for normal growth and development, but are toxic in surplus amounts. NGH in response to copper stress has previously been identified in wild yeast isolates, but this was not produced under standardised conditions. Can we reproduce the conditions from the environment to produce high NGH through a controlled experimental evolution?

Batch style evolution at 18°C occurred for ≥ 600 generations under three conditions (no stress, 6 mM copper nitrate stress, or 1 mM zinc chloride stress). Evolution under metal stress produced strains with increased metal resistance. Increased NGH is suggested to evolve in response to copper nitrate exposure, but not in response to zinc chloride exposure, though this change is not reproducable after a further 20 generations in the absense of stress. Flow cytometric determination of variation in Phloxine B staining under stress (a measure of cell vitality), revealed a) an increase in variation in absorbance among cells in cells evolved with copper stress compared to those that were not, and b) cell size variation showed no change**.** Colony size variation increased when cells were evolved with copper nitrate, driven by the appearance of large and small colony subpopulations. This change is not indefinitely stable**,** only remaining for around 30 generations. Small colonies may provide a fitness benefit to the population as a whole. Whole genome sequencing will reveal any SNP changes responsible for the phenotypes observed.

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INTRODUCTION

NON-GENOTYPIC HETEROGENEITY (NGH)

AN INTRODUCTION TO NGH

The basis for differences in behaviour between cells can be split into three broad categories; environmental, genotypic, and non-genotypic. In recent years there has been an increased focus on variation at an individual-cell level, rather than at a population level. There has been a flurry of research on a range of phenotypes and cell lines that are affected (yeast, bacterial and mammalian) (Acar *et al.,* 2008; Holland *et al.,* 2014; Levy & Siegal, 2008, Levy *et al,.* 2014). Individual cells can show variation in phenotype, despite being derived from an isogenic population. Although the cells all share a genetic background, they can show variation in a wide range of phenotypes. These include many phenotypes that are linked to essential cellular functions such as cell differentiation (Mirouze *et al,.* 2011; Buganim *et al.,* 2012) and pathogenic virulence (Halliwell *et al.,* 2012).

Genotypic variation is well studied. It describes a heritable change in DNA sequence within an individual's genome, which leads to a modification of one or more protein products. The modified product may have altered function, potentially causing a change in fitness. If the change confers an increase in fitness, there will be positive selection for the mutant phenotype, hence becoming fixed in the population according to natural selection (Darwin, 1859).

Phenotypic plasticity describes the ability of a population of cells to switch phenotype upon exposure to new environmental conditions (Via & Lande, 1985). Cells may be able to produce several different behaviours depending on the change. When the environment changes, the entire population may switch phenotype accordingly. Switching phenotype can be slow and costly.

Non-genotypic heterogeneity (NGH) should not be confused with phenotypic plasticity. NGH is not contingent on a change in environmental conditions, instead producing a range of phenotypes even under standard growth conditions. It is possible that certain environmental conditions alter the level of NGH observed in the same population. Thus, NGH may be considered to pre-equip the cellular population in anticipation of potential future environmental perturbations, without incurring the risk associated with slow and potentially-costly switching of phenotype. From a classical deterministic point of view, newly modified alleles become fixed in a population if they confer increased fitness. In a frequently fluctuating environment, individuals that can respond rapidly to new conditions will be selected for. Bet hedging by diversifying phenotypes is one strategy that benefits the population in this situation (Beaumont *et al.,* 2009). Although a large proportion of the population may be sacrificed, a proportion of the cells may be able to survive the new stress in order to re-populate the environment (persister cells) (Marcusson *et al.* 1999). This will therefore benefit the population as a whole. It is reasonable to predict that NGH can be selected for under appropriate evolutionary pressures, just as genotypic heterogeneity can.

Figure

BENEFITS OF EXHIBITING HIGH NGH

NGH may be observed in populations simply due to a high cost of suppressing noise (Lestas *et al.,* 2010). If this is the case, this would predict that a limited degree of variation in trait expression is unavoidable, and that if measured precisely enough all traits will vary in expression between cells to some extent. However, as outlined below, there have been a number of studies that have shown an adaptive benefit to exhibiting variation in a phenotype. In order to argue that NGH is functionally beneficial we must first address two questions; a) is the degree of variation observed above the lower limits mentioned above and b) does the variation observed serve to increase the probability of the population's survival?

There are a handful of studies that illustrate natural genetic variation in wild microbial populations that lead to differences in phenotypic heterogeneity under appropriate environmental conditions. Differing degrees of cell-to-cell variability in cellular morphology (Yvert *et al.,* 2013), and in stress resistance (Holland *et al.,* 2014) were observed between natural yeast isolates. Similar variation has also been identified in much more complex organisms, though the mechanisms are likely to be significantly more intricate, due to their larger genome. Natural stocks of *Drosophila melanogaster*, subjected to supervised crosses, showed an increased fixation rate of developmental asymmetry (Carter & Houle, 2011). In a separate study, natural fly genotypes that alter the number of sensory bristles were identified (Mackay & Lyman, 2005). The regulatory pathway leading to this phenotype is very complex, with a large number of genes affecting bristle number between individual flies, and is often affected by environment-specific signals. Environmental impacts on trait variation are not uniform across genotypes in either snails (Ros, et al., 2004) or maize (Ordas, *et al.,* 2008). A study on metabolic and transcriptomic variation in *Arabidopsis* species found multiple genetic loci conferring significant, heritable differences in stochastic noise. They were also able to validate effects of stochastic variation at *ELF3* on the transcriptional oscillations that take place during circadian cycles and metabolism (Jimenez-Gomez, *et al.*, 2011).

Environmental adaptation by microorganisms is traditionally viewed as the ability of populations to sense and respond to environmental cues. It is possible that if a population exists in an environment that is switching states at high frequency, signal transduction is simply not fast enough to elicit a response in time to allow survival. Maintaining high resonance phenotypes at a high frequency is costly, as seen by Acar *et al.* (2008). Although fast-switching phenotypes outgrow slow-switching phenotypes when the environment is rapidly fluctuating, a stable environment elicits the opposite response. Alternatively, as there may be many more possible environments than can be accommodated by an organism's repertoire of signal transduction

pathways and/or evolutionary history, so expression of a broad range of phenotypic states is therefore a possible strategy for improving fitness in a changing environment.

This strategy is known as bet-hedging and has been observed in a range of species (Philippi & Seher, 1989; Beaumont, *et al.*, 2009). In the case of adverse environmental conditions, most cells may not survive but a small proportion may express a particular phenotypic state, allowing them to survive the environmental perturbation; even though these may even grow more slowly under standard conditions if the phenotype is costly.

In addition to allowing the possibility of continuous ranges of phenotype, NGH promotes new functionality seen as distinct subpopulations (Johnson, *et al.* 2012). One would predict an increase in NGH such as this if there were a requirement for incompatible functionalities. Subpopulations can divide the labour. This type of cellular co-operation could provide insight into early stages of multicellular life and other ecological principles.

CONTEXT AND SIGNIFICANCE

NGH has the potential to play important roles in diverse aspects of microbiology including microbial ecology. It is paramount for species to have mechanisms that allow re-establishment of populations by maintaining a subset of individuals able to survive the onset of stressful conditions (e.g. changes in temperature, pH, nutrients, toxins).

The phenomenon also has practical applications to medical studies and within the food industry. There may be high variation in virulence phenotypes between cells, allowing enhanced pathogenicity or resistance to antimicrobial drugs or preservatives in certain individuals. The pathogenic yeast *Candida glabrata* undergoes phenotypic switching in response to copper stress (Srikantha, *et al.* 2005) at the site of infection (Brockert, *et al.,* 2003). High

heterogeneity in the expression of Epa1 was determined to be responsible for altering a virulence trait in *Candida glabrata* (Halliwell, *et al.,* 2012). Individual cells that are more highly resistant to anti-fungal agents are observed during biofilm formation in the human fungal pathogen *Candida albicans* (LaFleur, *et al.,* 2006). In a bacterial population, individual cells that have a slow growth rate have been found able to survive ampicillin antibiotic treatment (Balaban, *et al.,* 2004). Persister cells such as these have been observed in a range of pathogenic bacterial strains; e.g., *E. coli* (Marcusson, *et al.*, 2005), *Mycobacterium tuberculosis* (Wallis, *et al*., 1999), *Staphylococcus aureus* (Bigger, 1944), and *Pseudomonas aeruginosa* (Keren, *et al.,* 2004).

Looking at hetetrogeneous expression with human conditions with a genetic basis, the *Nf1* heterozygous mutation causes neurofibromatosis type 1 in humans with varying penetrance. Studies on these mammalian cell lines showed an increased morphological variability among cultured melanocytes from an individual donor (Kemkemer, *et al.*, 2002). Variation in expression of *FTO,* encoding the alpha-ketoglutarate-dependant dioxygenase protein, has also been associated with obesity and inter-individual variation of body mass index (Yang, *et al.,* 2012).

In the food industry, microorganisms that cause food spoilage pose a great threat. To minimise the problems associated with contamination (batch wastage, customer complaints etc.) companies invest in quality assurance protocols and the use of antimicrobial agents. Heterogeneity in the resistance of individuals to antimicrobials increases the chances of persistent cells surviving in foods that have been treated with such preservatives. The food spoilage yeast *Zygosaccharomyces baiili*, for example, exhibits high resistance to a range of weak acid preservatives such as acetic acid, benzoic acid, and sorbic acid (Stratford, *et al.*, 2013). Tolerance to the acidic conditions was only displayed by a small subset of the isogenic population that had lower internal pH, decreasing the cells' ability to accumulate the weak acids.

There are many examples in the literature of phenotypic heterogeneity allowing the survival of persister cells, for example in yeast after heat stress (Levy, *et al.,* 2012). Also, examples of individual cells responding differently to stress (Ni, *et al.,* 2012).

WHAT MECHANISMS LEAD TO OBSERVED DIFFERENCES IN NGH?

Heterogeneity in growth rates was observed in *E. coli* populations that were exposed to antibiotics (Balaban, *et al.* 2004). A small population of dormant cells were identified. The formation of the persistent population has been linked to fluctuations in the expression of HipA, in *E. coli* (Rotem, *et al.,* 2010). This protein forms part of the toxin-antitoxin *hipBA* module. Levels of toxin above a threshold value cause the cells to enter dormancy, and the extent to which the level exceeds the threshold determines the length of the response. As the level of toxin fluctuates, a mixed population of metabolically active and dormant, drug-insensitive cells is observed.

Another trait linked to increased fitness in a fluctuating stressful environment is variation in lag time before growth (Fridman, *et al.,* 2014). Cells in stationary phase at the time of exposure exhibit a delay in growth when the environment changes. By extending the time to first division the cell can maintain a relatively dormant state and avoid the harmful effects of the antibiotic. If extended lag time is the main factor determining tolerance, the effects should not be specific to a single stressful condition. Evolution of bacteria was conducted in an intermittently stressed environment, then the resistance and tolerance of the evolved strains were characterised. Although ancestral cells divided within half an hour, evolved cells had lag times that extended for many hours. Cells that were not allowed to reach stationary phase before exposure to stressor did not show increased survival. Under selection, strains had evolved specific genetic mutations that became fixed in the population and sequencing identified these. The mutations conferred a

survival advantage non-specifically against a broad range of stress and antibiotics. A similar response was seen in isogenic *Lobelia inflata* seeds which when exposed to environmental variability show longer germination times (Simons & Johnston, 2006).

MOLECULAR BASIS OF NGH

NGH may be caused by a variety of internal molecular biology mechanisms. Firstly, stochastic molecular processes such as gene expression noise, positive feedback loops which amplify variation, and processes with low molecular number are subject to high levels of variation, as are steps in reaction chains with slow reaction rates. Analysis across the yeast genome has identified that house-keeping genes, and genes with essential function have low levels of intrinsic variation in expression, while genes with products which are only transiently required (such as those needed for stress response) are subject to higher levels of expression noise (Bar-Even, 2006).

Periodic oscillations may also cause fluctuations in expression. This might include cell cycle (incorporating stochastic partitioning at division), ultradian rhythms, and circadian rhythms (Bass, 2012). Isogenic cultures contain a range of cellular ages, which may also cause observable differences in individual cell behaviour. Cellular communication may also be a determining factor. Neighbouring cells in the population may influence the decision of an individual cell similar to Quorum sensing as observed in bacteria (Rutherford and Bassler, 2012). Communication may be due to contact dependant signalling by membrane proteins, whereby signals can be shared within a population about environmental queues, and population density.

Specific DNA mutations may be responsible for the aforementioned changes. Any mutations that lead to an increased rate of phenotype switching due to increased efficiency of a feedback loop, or which occur within a regulatory region of gene expression may increase rates of NGH (Acar, *et al*. 2008).

Epigenetic alterations can equally be added to a regulatory region of a gene to the same effect (Davis. *et al.*, 2013).

An increase in NGH can be seen both in discrete traits, and in continuous traits. For discrete traits, an acquired mutation may increase the rate of switching between an “adapted” to a “non-adapted” state (for example in the case of increased efficiency of a feedback loop). For quantitative traits, individual cells may have altered variance in a single trait. This could be due to changing co-operativity at promoters (Becskei, *et al*., 2005), modulation of chromatin dynamics (Raser & O'Shea, 2014), transcriptional elongation (Ansel, *et al.,* 2008), or translational efficiency (Guido, *et al.*, 2007).

It is important to determine if alleles conferring increased phenotypic heterogeneity segregate naturally in wild populations. If this is the case then evolution can act upon these alleles. Fehrmann *et al.* (2013) carried out genetic mapping of three genetic loci whose presence increased phenotypic variability in expression of a GFP tagged reporter. When the three loci were established in a single strain, the increase in variability was greater, showing an additive effect. Allele-replacement experiments confirmed that two naturally occurring transmembrane transporters (*ERC1* and *MUP1*) were responsible for the increase. Their results also suggest that changes act on specific pathways, rather than pleiotropically. *MUP1* has previously been ranked as the noisiest plasma membrane transporter gene, at 4.7 times greater variation than average (Zhand, *et al.,* 2009).

High variability phenotypes are often not fit in fixed environments, or rapidly switching environments as the switching rate between phenotypes may be too slow. In a population with two environmental states, and two phenotypic states; slow switchers in a fast changing environment will be more homogeneous (more are killed) while fast switchers in a slow changing environment will be more heterogeneous (more are able to survive). After transition to a new environment, fast switchers have a higher growth rate

than slow switchers in the initial growth rate, but slower growth rate once established (Acar, *et al.,* 2008; Stomp, *et al.,* 2008).

METHODS FOR MEASURING NGH

The ability to examine single cells microscopically has been available for many years, but the information that can be gleaned from this type of methodology is limited. as it looks at the population as a whole. In order to study microbes at a single cell level, the field needed novel methodology to accompany the novel thinking. Single molecule and cellular variation has been measured using a variety of methods in previous research, a few of which are covered below. It should be noted that the shape of the distribution could also give important information about a trait. Depending on evolutionary history and the mechanisms, leading to the increase in NGH, distributions may increase in diversity in a normal fashion, as well as in a non-normal skewed fashion. Some distributions may become bimodal (Martins and Locke, 2015).

When exposed to stress, colony forming ability indicates the viability of individual cells within a culture. Therefore, by exposing an isogenic population to a range of concentrations of the stressor of interest, a “kill curve” comparing the ability for single cells to survive can be produced (Figure 1). The gradient of this curve describes the extent of heterogeneity specific to that stress (Holland, *et al.* 2014; Stratford, *et al.,* 2013) where a steep curve indicates little heterogeneity in resistance to the stress between individual cells, while a shallow curve indicates high heterogeneity. The tail end of this curve represents rare persistors within the population.

The methodology of flow cytometry is inherently suited to the study of NGH, and is becoming more commonplace in the context of studying single cell variation. By using a variety of fluorescent probes, it is possibly to assess many aspect of cell physiology. An example of such a dye is the vitality stain, Phloxine B (Noda, 2008). The stain is actively pumped out of ‘healthy’ cells,

and as the cell becomes increasingly unhealthy, it is less able to meet the energy demands, and therefore contains more of the stain in the cytoplasm. A wide distribution of dye intensities would indicate a high heterogeneity response to stress, while a narrow distribution would indicate a low heterogeneity. Other dye methods may measure live/dead ratio within a population (Davey, *et al.*, 2004; Guyot, *et al.,* 2015) by exposing the culture to an increasing intensity of stress, the ratio can be used to create a killing curve of which the gradient can be used as with the kill curve assay on plates.

High-throughput microscopy has been used to measure various aspects of cellular behaviour, for example protein expression, morphology, and growth rate (Levy & Siegal, 2008). Survival outcomes relating to these original measurement also gives more insight into their impact. Using an automated system, cells can be tracked through from single cell stage through to microcolony.

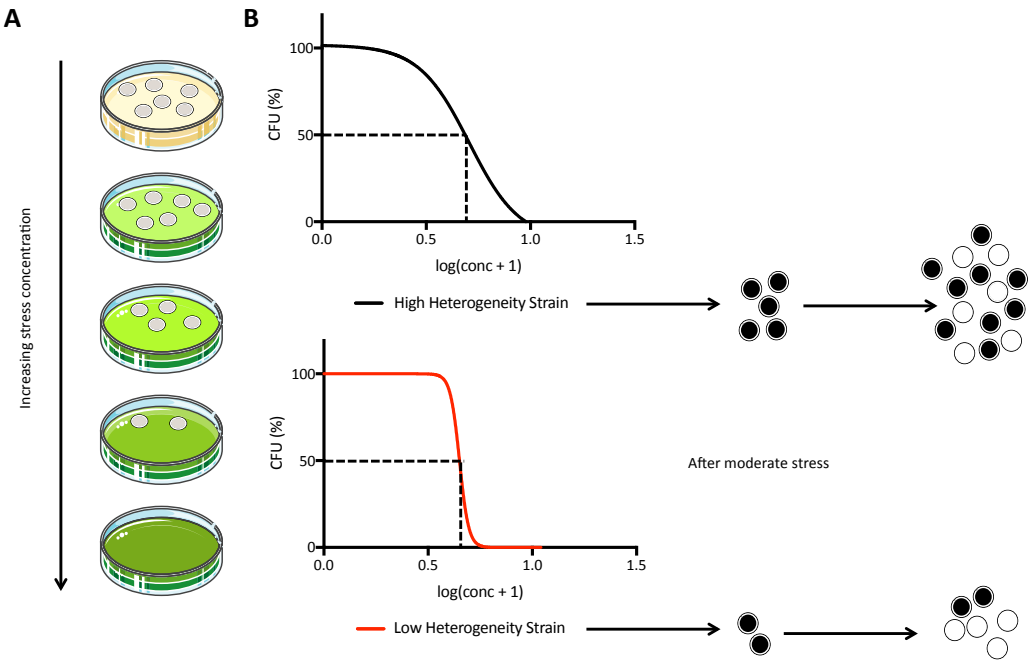

Figure 1. Kill curve assay methodology. **(A)** Plates containing agar medium inoculated with a increasing level of stress concentration resulting in increasingly few colonies able to form colonies. **(B)** Resulting kill curves produced from plate series, where 100% indicates the number of CFUs on 0 mM stress, and the concentration it logged prior to plotting.

EVOLUTIONARY FORCES

A BRIEF HISTORY OF EVOLUTIONARY STUDIES

Traditionally studying evolution was a difficult task. The main method for determining the consequences of this important process was using comparative studies, comparing the living organisms on earth today with the fossil record. Molecular advances have allowed for more in depth, and accurate, phylogenetic studies.

Where comparison to the fossil record is not possible, it is possible to examine the closest living relative. Darwin (1859) stated that *"in looking for gradations by which an organ in any species has been perfected, we ought to look exclusively to its lineal ancestors; but this is scarcely ever possible, and we are forced in each case to species of the same group, that is to the collateral descendants from the same original parent-form".* We use phylogenetic similarity because in many cases we have to though critical conclusions about the mechanisms are lacking from such methods. There would be much more value in observing the process of natural selection directly, determining the process of adaptation by descent, and using microorganisms is one way of observing evolution first-hand, and determining this key information.

Fruit flies were the main subject of traditional experimental evolution, starting with Morgan (1911) and continued through many decades through many population geneticists. Some groups used bacteria (Atwood, *et al.,* 1951) but this didn't take hold for many years. The study of microorganism evolution has become more common in recent years, however, with evolutionary ecologists realising the need for rigorous hypotheses testing, and microbiologists realising the importance of an evolutionary perspective. The increase in popularity of experimental evolution using microorganisms was partly due to an increased ease of collecting genomic data. It was obvious that experimental evolutionary approaches could open the door on many scientific

advances. Bacteria, viruses, unicellular algae, and fungi have now all been used for microbial evolutionary experiments (Hedges, 2002).

USING MICROBES IN EVOLUTIONARY STUDIES

Microbes enable the study of the reproducibility of evolutionary outcomes. Genetically identical cells can be found in separate populations under identical environmental conditions. Naturally, these situations could be due to mutations in the founding population, or in subtle variation in environmental conditions, but without rigorous testing it is impossible to distinguish between the two. It has been experimentally shown that separate populations can produce very different evolutionary outcomes (Lenski, *et al.,* 1991; Vasi, *et al.,* 1994). A frozen record of the ancestral population, along with samples from varying time-points throughout the evolution, and a final "evolved" strain can all be collected and compared for fitness both genetically and phenotypically.

Benefits of using microbes over multicellular organisms include the ease in propagation and enumeration, fast generations time, ability to store large populations in a small space, to store samples almost indefinitely and revive reliably. In addition to this, it is easy to conduct experimental replication, impose specific environmental parameters. Generally, there is abundant genetic information available for many species. It is generally easy to manipulate the starting composition of the population, and asexuality maintains linkage between a genetic marker and the genomic background in which it is placed. In the interest of studying NGH, ability to produce generically identical populations is essential. The fact that microbial division is asexual, unlike most plants and animals, is key (Yang and Kim, 2016).

Although many microbes act as pathogens to humans, they can also be non-harmful symbionts (Hooper et al., 2002). They are also essential for many ecosystem services, though they can come at a cost (eg. mycorrhizal

associations (Johnson *et al*., 1997)). We should therefore understand the mechanisms and dynamics of their evolution.

GENETICS IN AN EVOLVING POPULATION

It is widely accepted that beneficial mutations will become fixed in a population via selection of the fittest. However, beneficial mutations can be lost by random drift, especially in small populations. Lenski *et al.* (1991) describes the likelihood that a beneficial mutation survives extinction by random drift is twice its selective advantage.

Gerrish & Lenski (1998) coined the term "Clonal interference". In sexual populations, recombination allows beneficial mutations from separate lineages to combine in a fitter, double mutant. In asexual populations, clones must compete until fixation – a slow process in which one of the beneficial mutations is lost. Beneficial mutations are therefore acquired in a step-wise manner. Clonal interference theoretically has several consequences for asexual population genetics. As population size increases, the probability of substitution of a beneficial mutation decreases, but successful mutations should entail a greater fitness benefit. The rate that the mutation spreads will be reduced compared to predictions based purely on its fitness advantage. There are likely to be many transient beneficial mutations within the population, but these will eventually be lost. Finally, the current dominating genotype may not be most closely related to the previously dominating genotype, rather to a more distant. These effects have been experimentally supported in laboratory bacterial populations (de Visser, *et al.*, 1999; Imhof & Schlötterer, 2001; Rozen, *et al.,* 2002; Shaver, *et al.*, 2002).

Microbial strategies to increase survival fall into two categories; increased resistance, and increased tolerance. Resistance allows the microbe to survive in persistent presence of the stress, while tolerance allows survival through exposure to high levels of stress, but only for limited periods of time (Cohen, *et al*. 2013). Tolerance is better understood than resistance, despite both

being important for the failure of antibiotics in the treatment of human diseases. Resistance can be tested using MIC tests (minimum inhibitory concentration, the highest concentration that the strain can be exposed to without showing inhibition), while tolerance can be tested using the MDK_{99} (the minimum duration needed to kill 99% of the cells). A long MDK_{99} refers to strains that require longer treatments to attain the same level of killing (Fridman, *et al*. 2014).

Experimental evolution can be supplemented with a quantitative measure of fitness to add valuable insights. Fitness could be measured by the ability to propagate descendants in comparison to the ancestral strain using a competition study such as that used by Lenski *et al.* (1991). Genetic marking allows the relative quantification, and measures relative population growth rates under competition for a limited resource.

Fitness increase is rapid initially, and then slows, indicating a movement towards an adaptive peak. For example, *E. coli* increased 10 fold more in the first 5000 generations of experimental evolution, than between 15,000 and 20,000 generations. Fitness was still increasing at 20,000 generations however, so the population had still not reached maximum fitness (Cooper & Lenski 2000).

METAL STRESS

We already know that individual variation is displayed in a variety of traits, including several that are essential for survival (eg virulence, antibiotic resistance). Wild population of yeast were tested for heterogeneity in resistance response to trace metals (Holland, *et al.,* 2014).

Copper can adopt distinct redox states, and it can be found in its oxidised state [Cu(II)], and reduced state [Cu(I)]. As copper is so highly reactive, organisms ranging from bacteria to large, multicellular mammals utilise this trace element as an important catalytic co-factor for many protein pathways that are essential for normal growth and development (Graden & Wings, 1997). Superoxide dismutase that is involved in defence against oxidative damage, cytochrome oxidase that is involved in respiration, and Fet3p ferro-oxidase that is linked to iron uptake, are just a few of the key enzymatic processes in yeast in which copper is essential (Graden & Wings, 1997). It is, however, toxic to cells if accumulated to a level surplus to requirement. Copper can displace other essential metal co-factors in proteins, preventing key reactions, but due to its reactive nature also produces highly damaging oxygen species (such as hydroxyl radicals) (Peña, *et al.,* 1999). It has previously been shown that direct exposure of DNA to copper resulted in a higher than expected mutation rate (Tkeshelashvili, *et al.,* 1991), and a micronuclei method which accurately detects errors in chromosome segregation during mitosis also found that copper acts as a mutagen in mice tissue (Pra, *et al.,* 2008). Copper also has the advantage of being a major pollutant in the natural environment of sticklebacks in Scotland and gives a great opportunity to attempt to apply our research to multi cellular organisms.

Unlike copper, zinc only be found in the Zn^{2+} state, making it redox non-reactive. It is in fact known to function in a number of antioxidant pathways (Powell, 2000). Zinc also acts as a co-factor to many proteins in a range of

processes, for example superoxide dismutase and metallothioneins which protecting from the effects of other metals. A large proportion of the zinc tolerance related genes function in general stress response, or vacuolar assembly and maintenance, for example vacuolar zinc transporters ZRC1 (MacDiarmid, *et al.,* 2003), and ZRT1 (Gitan, *et al.,* 1998).

PROJECT AIMS

Here I will be conducting an evolution experiment to complement previous work conducted in the host lab. Previous work (Holland, *et al*., 2014) showed that NGH is widespread in wild yeast populations. Three dfifferent yeast species were examined; *C. podzolicus*, *C. sake* and *S. roseus.* They found that isolates with the highest level of NGH originated from environments with high levels of pollution, while isolates with the lowest level of NGH originated in unpolluted nearby locations. Furthermore, those isolates of *C. sake* which had been identified to have with high NGH displayed increased levels of fitness than isolates with lower heterogeneity when exposed to lead, which they had previously been exposed to in the wild. This supports the hypothesis that NGH confers a survival advantage under certain conditions.

Wild isolates of *C. sake* were than evolved under controlled evolutionary conditions, demonstrating that NGH could be selected for during long-term environmental stress of wild microbial isolates.

The evolution carried out here will evaluate if exposure to stress for a minimum of 600 generations (specifically exposure to trace metals copper and zinc) leads to an increase in NGH in the well studied model yeast strain *S. cerevisiae*, as predicted by these earlier findings. This will be tested using increasing concentrations of the metal on agar plates and counting the colony forming units. If an increase in NGH is identified, I will ascertain which genes were responsible for the increase by comparing the well-documented genome to isolates from a culture that was evolved for an equal period but without stress, and to the parental WT strain.

MATERIALS AND METHODS

YEAST STRAINS, MEDIA AND GROWTH CONDITIONS

The wild-type (WT) haploid strain *Saccharomyces cerevisiae* BY4741 was used for all experimental evolution studies. The WT strain was obtained previously by the host laboratory (Euroscarf, Frankfurt).

Unless otherwise stated, the following steps were taken to prepare cultures for experimentation. Strains were stored as primary stocks at -80°C in 30% (v/v) glycerol. Prior to use, YEPD agar medium (peptone 20 gL^{-1}, yeast extract 10 gL^{-1}, agar 16 gL^{-1}, 2% glucose) was inoculated with the strain to allow isolation of single colonies for experimentation. Pre-cultures were created by picking single colonies suspending them in 10 ml YEPD broth medium (peptone 20 gL^{-1}, yeast extract 10 gL^{-1}, 2% glucose). Conical flasks were autoclaved before use, sealed with a foam bung during growth, and filled with medium to 20% volume of the 50 ml flask capacity for comparable conditions between experiments. This 'pre-culture' was grown overnight (24°C, 120 rpm). Cultures were then diluted to an OD_{600} of 0.5 (approx. 1.5 x 10^7 cells/ml) and left to recover (24°C, 120 rpm) for a further 5 hours. This typically allows for two cell divisions under these conditions. After this period cultures were still within the exponential phase upon dilution for experimental purposes.

Where specified, YNB broth medium (YNB 6.9 gL^{-1}, 2% glucose, agar 16 gL^{-1}) and YNB agar medium (YNB 6.9 gL^{-1}, agar 16 gL^{-1}, 2% glucose) were used in place of YEPD broth.

It should be noted that there will be at least 15 generations between freezer stock revival and any assay.

PRELIMINARY INVESTIGATION OF CONDITIONS FOR EXPERIMENTAL EVOLUTION

In order to avoid potential non-specific effects of differential entry/exit from stationary phase in parallel experimental evolution assays, experiments were designed so that cultures remained in exponential phase throughout the experimental evolution. To help ensure this in batch culture (i.e., preclude overgrowth between each subculture), a variety of conditions were tested to reduce growth rate from the standard 90 minute cell doubling time, as calculated in Figure 2. Single colonies of BY4741 were used to inoculate 10 ml YEPD broth or YNB broth. These were grown at 18°C or 30°C. Conditions selected from these results were then used to assay *S. cerevisiae* growth in the presence of a range of copper concentrations to determine appropriate concentration for the evolution.

$$\text{doubling time (hrs)} = \frac{-0.301 \times \text{time period (hrs)}}{\log(\text{initial OD}_{600}) - \log(\text{final OD}_{600})}$$

Figure 2. Equation to determine doubling time from a growth curve.

EXPERIMENTAL EVOLUTION OF YEAST V I

A single colony of BY4741 was grown overnight (24°C, 120 rpm) in 10 ml YEPD (2% Glucose), at 20% volume of the flask capacity. After 16 hours, the culture was split into 30 flasks, and diluted to a final OD of 0.1.

The aliquots were made up to 50 ml volume, in 250 ml conical flasks. Cells were cultured at 18°C with vigorous rocking. Cultures were consistently stored in 50 ml liquid broth, comprising 20% volume of a 250 ml conical flask. Every

2-3 days the cells reached a final population size of approximately 1.5 x 10^8 cells/ml, and were then sub-cultured into fresh medium, undergoing a minimum bottleneck of 100,000 cells. Three separate experimental conditions were maintained throughout a minimum of 600 generations; unstressed, 6 mM copper nitrate stress (Cu), and 1 mM zinc chloride stress (Zn). After approximately 300 generations, the concentration of copper nitrate was increased to 6.5 mM due to an observed increase in doubling time (noticeable by density of culture increasing over the same time period). This indicated that cells were under continuous exposure to the stressor.

EXPERIMENTAL EVOLUTION OF YEAST V II

Single colonies of evolved BY4741 from the populations described above were grown in 24 hours intervals (24°C, 120 rpm) in 10 ml YEPD (2% Glucose) with no stress, at 20% volume of the 50 ml flask capacity. Once per day, a 1 ml aliquot was frozen as previously described, and 100 µl was transferred to 9.9 ml of fresh media. 4 subcultures equates to around 60 generations, around 15 generations per day.

DETERMINATION OF METAL RESISTANCE AND HETEROGENEITY

YEPD agar (2% glucose) was melted using a microwave at medium power. This was cooled to 60C in a water bath, and then measured into a new bottle along with stressor solution at the desired concentration. Stressor solution was added at 20% of the total volume of agar. After mixing well, approximately 25 ml of agar was poured into 9 cm petri dishes and air-dried until set. Lids were then replaced and plates were stacked for a maximum of 24 hours before use to avoid evaporation effects on the concentration of stressor.

Exponential phase cultures were diluted to an OD_{600} of 0.1 before serial dilution to a final cell density of ~3,000 cells/ml (OD_{600} ~0.0001). Approximately 300 cells were then aliquoted onto the prepared 9 cm petri dishes.

Once the cultures were spread, plates were left to air dry for a minimum of 10 minutes before stacking. For storage, plate stacks were wrapped in plastic plate bags, taped, and stored in a box at room temperature for 14 days before counting. This procedure reduced the extent of evaporation, and minimised any variation in oxygen availability within a stack.

Colonies on plates were counted using an automated program powered by Protos 3™ (Don Whitley Scientific). The data was exported in a .csv format for further processing using Excel. Curves were standardised so that 100 % survival was equal to the number of colonies present when the culture was exposed to no stress. To standardise the weighting at 0 % survival for plot fitting, data points past the first concentration at which no colonies were counted (MIC) were discounted and two further points were added at the MIC + 10% and 20% respectively.

For each data set, a sigmoidal curve was fitted to the data using Prism software. This determines IC50 and Hillslope values (gradients) for the curves. For ease of analysis, the log of the modulus of the Hillslope was used as the measure for heterogeneity, where the gradients of kill curves reflect population heterogeneity of stress resistance (Sumner, *et al*., 2003). The IC50 was used as the measure for population-averaged resistance.

CELL SIZE COMPARISONS BY FLOW CYTOMETRY

Cultures were diluted to OD_{600} 1 in YEPD (20% Glucose). 500 µl aliquots consisting of 472.5 µl of culture, 25 µl stressor solution, and 5 µl Phloxine B (final concentration 5 µg/ml) were produced for each condition in 2 ml

Eppendorf tubes. These tubes were incubated in the dark at 120 rpm, at 24°C, for 1 hour. For each strain, conditions included a non-stained control, a range of stained stressor concentrations, and a heat killed stained population.

After incubation, tubes were microcentrifuged at 1200 rpm for 60 seconds. The supernatant was removed and the pellet resuspended in 100 µl MES buffer. The sample was centrifuged at top speed for one further minute. The supernatant was again removed, and the pellet resuspended in 500 µl MES buffer. The whole sample was then transferred into FACS tubes for flow cytometric analysis on Becton Dickinson LSR11. A minimum of 10,000 events were measured for each sample. The sample was vortexed prior to taking each measurement. Calibration settings were as follows: FSC 360, SSC 325, PE 560 volts, PE-A ratio 70%.

Data were analysed using Weasel software. Three gates were applied before exporting statistics from the data set. Cells that were not within the main population for size were excluded, along with doublets and those that fall within the dead cell population defined from the heat killed control.

COLONY SIZE VARIATION

Cultures were diluted to an OD_{600} of 0.1 before undergoing a serial dilution to a final cell density of ~1,000 cells/ml (OD_{600} 0.00003). Approximately 50 cells were then aliquoted to YEPD agar (2% glucose), prepared as described above.

Once the cultures were spread, plates were left to air dry for a minimum of 10 minutes before stacking and incubation as described above. For storage, plate stacks were wrapped in plastic plate bags, taped, and stored in a box for 2 weeks

After 3 days, colonies were analysed using an automated program powered by Protos 3™ (Don Whitley Scientific) to measure the area of the individual colonies. The mean size and co-efficient of variation (CV) were calculated for

each plate using Excel. CV is a measure of variation which is calculated by dividing the standard deviation (SD) by the mean. In this instance, the CV of the colony size area was used as the measure of heterogeneity.

ASSAY FOR PETITE MUTANTS

Petite mutants lack the ability to respire aerobically (Ferguson and von Borstel, 1992). To identify these petite mutants that typically have endured damage to the mitochondrial genome, or lack mitochondria altogether, two methods were used. A tetrazolium chloride (TTC) assay tests for respiratory activity required 20 ml melted TTC agar (sodium phosphate 9.51 gL^{-1}, TTC 1 gL^{-1}, agar 15 gL^{-1}) to be poured on top of previously formed colonies on YEPD agar. A red colour indicates respiratory activity. Petite mutants are identifiable by remaining white. Wild type colonies are red.

A second method was to used to identify petite mutants: YEPD media containing 2% glycerol, a non-fermentable carbon source, were inoculated in the same way as described above, followed by examination for growth where any growth on this medium would require mitochondria-dependent respiration.

SPOTTING ASSAY OF GROWTH

YNB agar (2% glucose) was melted using a microwave at medium power and a sub-inhibitory concentration of stressor was thoroughly incorporated by inversion. Approximately 25 ml of media per plate was poured into 9 cm Petri dishes that were air dried until set. Cultures were diluted to an OD_{600} of 2 (6 x 10^7 cells/ml) before 1 in 10 serial dilutions to a final cell density of 1,000 cells/ml (OD_{600} 0.00003). Aliquots (3-5 µl depending on the hydrophobicity of

the stressor used in the media) were pipetted onto the media. Images were captured after 3-4 days incubation.

gDNA extraction

Cultures were grown in 10 ml YEPD (2% glucose) for 24 hours (24°C, 120 rpm) in 50 ml falcon tubes to reach a high cell density. Cultures were centrifuged at 3,500 rpm for 10 minutes to pellet the cells. In 2 ml Eppendorf tubes, 0.3 g acid wash beads, the cell pellet, 200 µl Triton/SDS solution and 200 µl phenol-chloroform were vortexed for 2 minutes at high speed to lyse the cells. The mixture was then microcentrifuged for 15 minutes (14,000 rpm) to separate the organic layer from the DNA. DNA in the aqueous layer was transferred to a new tube. An equal volume of phenol chloroform was added to the new tube and vortexed for a further 2 minutes at high speed. After another 15 min microcentrifugation at 14,000 rpm, the aqueous phase was again transferred to a new tube. DNA was precipitated using 0.53 volumes of 70% v/v isopropanol. This mixture was frozen at -20°C for a minimum of 30 min before centrifuging the tube (14,000 rpm, 10 min). The DNA pellet was visible at this point in the protocol. The pellet was washed using 70% v/v ethanol. Effort was made to remove as much ethanol as possible manually, followed by air drying of the pellet to remove final traces. The pellet was suspended in TE (Tris/EDTA elution buffer, pH 8.5) for storage and transport.

Whole genome sequencing

DNA samples intended for whole genome sequencing were tested for integrity and for being contaminant-free by running on a 2% agarose gel, along with a Quick Load Purple 1 kb DNA ladder (NEB #N3232). Genomic DNA was visible as a single prominent band >12 Kb. The presence of more than one band or a smear could suggest the DNA may be degraded or have a contaminant such as RNA, which usually appears as a smear <200 bp. Due to

the presence of multiple bands in samples, these were subsequently treated with Ribonuclease A from the bovine pancreas (Sigma Aldrich R4642 10 mg/ml) to remove any traces of RNA. This resulted in the presence of a single band on the gel re-run. Concentration and quality of the DNA was further confirmed using NanoDrop measurements, aiming for values ≥1.80 of both $A_{260/230}$ and $A_{260/280}$ ratios.

Samples were sent to Centre for Genomic Research, Liverpool for sequencing. At the facility, low fold coverage re-sequencing of 23 yeast strains involved preparation of 23 fragment libraries using TruSeq Nano DNA libraries from the supplied yeast genomic DNA. Paired-end sequencing of the 23 indexed libraries was performed by running in a single lane of the Illumina HiSeq platform, generating in excess of 240M mappable reads per lane. Coverage was in excess of 20X. Centre for Genomic Research also performed the post-processing of reads, including quality control and transfer of fastq files.

De novo genome assembly involved the preparation of Illumina libraries from the supplied genomic DNA using the Illumina TruSeq (insert sizes: 200-300bp and 500-600bp) of the barcoded and indexed libraries on the Illumina MiSeq platform, generating data off in excess of 12M clusters. Centre for Genomic Research finally conducted post-processing of data, including QC, resolution of indexes and transfer of fastq files.

EVOLUTION OF *C.ELEGANS*

The nematode WT *C. elegans* strain, along with methodology, was obtained from David de Pomerai, within the University of Nottingham. The worms were cultivated for 80 weeks at 18°C, equating to approximately 160 generations, in 90 mm petri dishes containing Nematode Growth Medium (NGM) (NaCl 3 gL^{-1}, Peptone 2.5 gL^{-1}, agar 16 gL^{-1}, 1M calcium chloride 1 mlL^{-1}, 1M magnesium sulphate 1 mlL^{-1}, 1M potassium phosphate (pH 6) 25 mlL^{-1}). The agar was supplemented (or not) with 0.1 mM copper nitrate and 1 mM zinc

chloride. The *C. elegans* were therefore exposed to continuous stress. Growth was in the presence of a lawn of *E. coli* strain OP50 previously cultivated for 24 hours (37°C, 120 rpm) to provide a suitable supply of food for *C. elegans*. Once a week, 1 cm^2 agar on which nematodes had grown was transferred to a fresh plate using sterile technique.

C. elegans were frozen at the final time point by transferring 1 cm^2 agar to 'starvation plates' (NGM medium with no OP50 lawn) for 1 week, then adding approximately 5 ml freezing solution per plate (glycerol 300 gL^{-1}, NaCl 5.84 gL^{-1}, 50 mM potassium phosphate buffer (pH 6.0) chilled to 4°C, plates were agitated to dislodge worms from the agar surface, and these were transferred to Falcon tubes.

RESULTS

TESTING AND SELECTION OF APPROPRIATE PARAMETERS FOR EXPERIMENTAL EVOLUTION OF YEAST

Preliminary investigations with wild type *S. cerevisiae* BY4741 involved the production of growth curves under a range of conditions to determine appropriate parameters for planned experimental evolution studies. Firstly, media type and incubation temperature were considered (Figure 3A, B, Table 1). While *S. cerevisiae* is routinely cultured at 30°C in research laboratories, 18°C is closer to realistic environmental conditions. YNB media has a more precisely defined composition than YEPD but is less favoured by yeast as reflected by the longer doubling time during exponential phase (Table 1). The longer lag time observed at 30°C simply reflects differing starting ODs required to ensure both cultures entered exponential growth after approximately equal time. A slower growth rate, measured here as doubling time, would also help ensure that populations could be maintained longer in exponential phase between subcultures of batch growth throughout experimental evolution. Exponential phase doubling time at 30°C was 47% of that at 18°C in YEPD, and in YNB at 30°C was 55% of that at 18°C.

As temperature had the biggest impact on growth rate, whereas using YNB versus YEPD had a comparatively small effect at the lower temperature, the more appropriate conditions for the experimental evolution (in order to maximize the interval between subcultures while avoiding entry to stationary phase) were concluded to be growth at 18°C in YEPD medium.

For experimental evolution under metal-selection, the aim was to attain a degree of metal stress that only slightly inhibited growth rate over the long-term exposure. Automated plate readers were able to test a wider range of concentrations in a more high-throughput manner, so concentration tests were conducted at using this method. Unfortunately this came with the

caveat that tests would be conducted at 30°C, but would never the less give a good idea about suitable stress levels. Too much stress could create a genetic bottleneck, leading to a less fit population (Amos & Harwood, 1998). Copper nitrate concentrations ranging from 4 to 10 mM were tested (Figure 4A, Table 2), along with zinc chloride concentrations ranging from 2 to 10 mM (Figure 4B, Table 3). The results indicated that 7 mM copper nitrate extended the doubling time by 38%, and 2 mM zinc chloride by 92%. Therefore, for experimental evolution, slightly lower doses of 6 mM and 1 mM were chosen respectively.

After approximately 300 generations, the concentration of copper nitrate was increased to 6.5 mM due to an observed increase in doubling time (noticeable by density of culture increasing over the same time period).

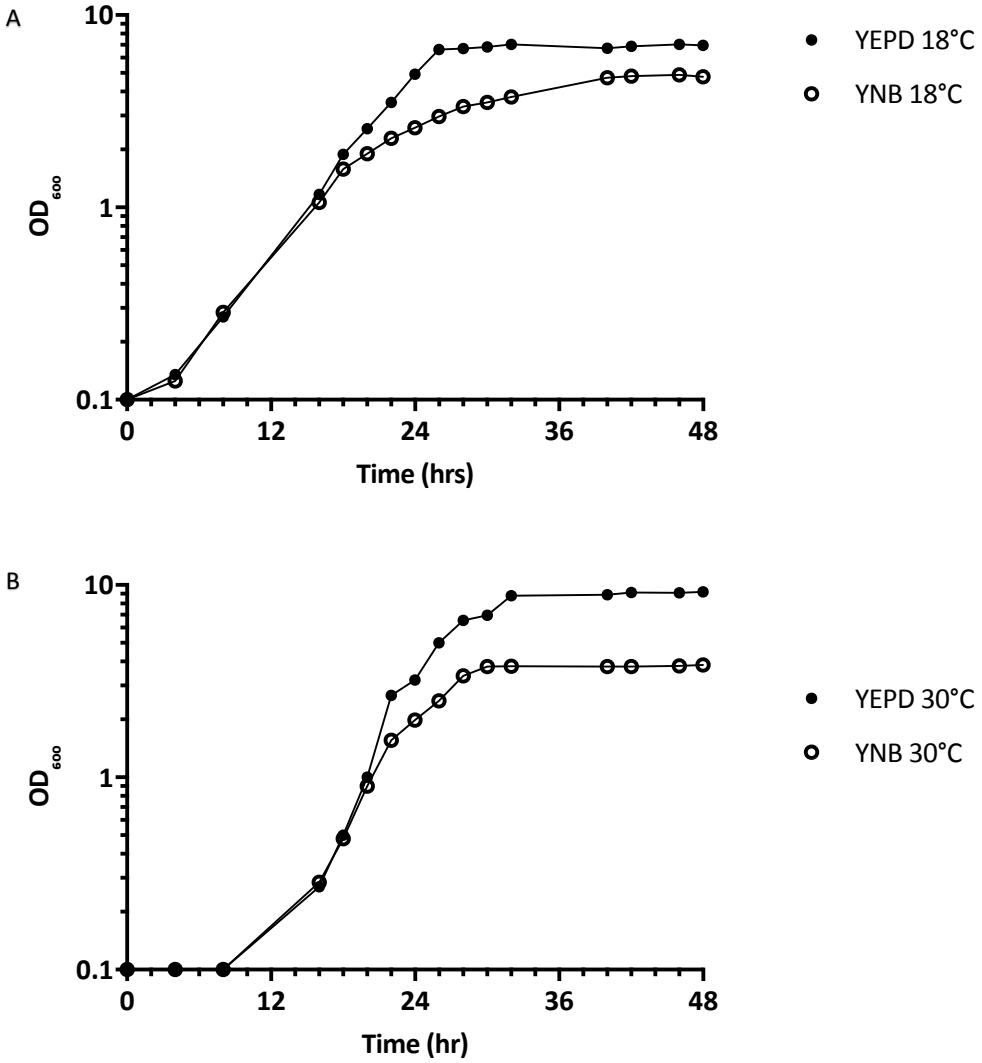

Figure 3. Growth curves of WT *S. cerevisiae* BY4741 grown in either YEPD or YNB medium at either **(A)** 18°C or **(B)** 30°C. Curves correspond to a single representative example of biological triplicates tested on separate days. The longer lag time observed at 30°C simply reflects differing starting ODs required to ensure both cultures entered exponential growth after approximately equal time.

Table 1. Doubling times determined from the growth curves observed from growing *S. cerevisiae* BY4741 in either YEPD or YNB medium at either 18°C or 30°C. Values correspond to the single representative example of biological triplicates tested on separate days shown in Figure 3.

Temperature (^{o}C)	Media	Doubling Time (hrs)
18	YEPD	3.8
18	YNB	4.4
30	YEPD	1.8
30	YNB	2.4

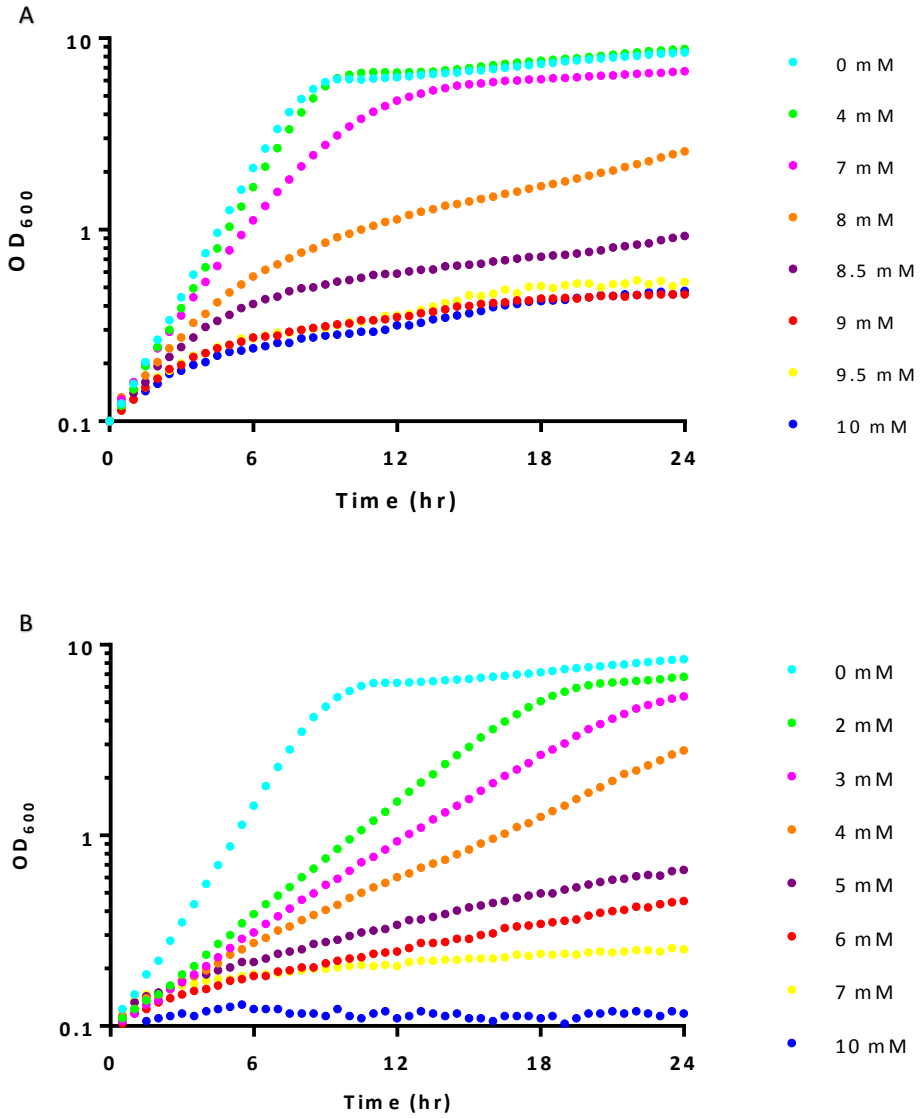

Figure 4. Growth curves of WT *S. cerevisiae* BY4741 grown in YEPD medium at 30°C exposed to a range of concentrations of **(A)** copper nitrate or **(B)** zinc chloride. Values correspond to a single representative example of biological triplicates tested on separate days.

Table 2. Doubling times determined from the growth curves observed from growing *S. cerevisiae* BY4741 exposed to a range of concentrations of copper nitrate. Values correspond to a single representative example of biological triplicates tested on separate days.

Copper Nitrate Concentration (mM)	Doubling Time (hr)
0	1.35
4	1.44
7	1.87
8	3.08
8.5	4.96
9	7.41
9.5	7.94
10	8.39

Table 3. Doubling times determined from the growth curves observed from growing *S. cerevisiae* BY4741 exposed to a range of concentrations of zinc chloride. Values correspond to a single representative example of biological triplicates tested on separate days.

Zinc Chloride Concentration (mM)	Doubling Time (hr)
0	1.47
2	2.82
3	3.41
4	4.20
5	9.28
6	8.79
7	18.71
10	50.97

EVOLUTION FOR ≥ 600 GENERATIONS UNDER METAL STRESS PRODUCED STRAINS WITH INCREASED METAL RESISTANCE

A single WT *S. cerevisiae* BY4741 colony was selected and grown overnight in YEPD agar before being aliquoted into 10 replicate colony lines per condition. The cells were then evolved in batch culture for a minimum of 600 generations in YEPD with known metal concentrations at 18°C. Cultures were maintained in exponential phase throughout by subculturing (to minimum starting population of 100,000 cells) twice weekly, before they could reach stationary phase. Culture aliquots were frozen at intervals during the experiments. It was likely that, as evolution progressed, the population was no longer isogenic but contained multiple evolved lineages. Therefore, for testing purposes, samples of the culture aliquots were streaked onto YEPD agar to enable isolation of single clones (see figure 21 for details).

Single colonies were assessed for resistance to the relevant metal used for selection during evolution. Colonies evolved with copper nitrate stress showed an elevated resistance to copper nitrate both when tested as mixed lineage background (i.e., aliquot from entire culture, rather than after individual-colony isolation; Figure 5A) and as single colonies (Figure 5B). Single clones isolated after evolution with copper nitrate displayed an IC_{50} of 11.7 ± 0.3 mM, significantly increased compared to the parental WT (9.7 ± 0.366 mM) and compared to the WT evolved in parallel without copper (9.5 ± 0.289 mM) (one way ANOVA, $F(2, 63) = 17.96$, $p < 0.0001$). Similarly, single clones isolated after evolution with zinc chloride showed increased resistance to zinc (8.6 ± 0.6 mM) (Figure 6), compared to the parental WT (7.1 ± 0.6 mM), and the WT evolved without zinc (7.0 ± 0.4 mM) ($t(8) = 2.786$, $p = 0.0237$).

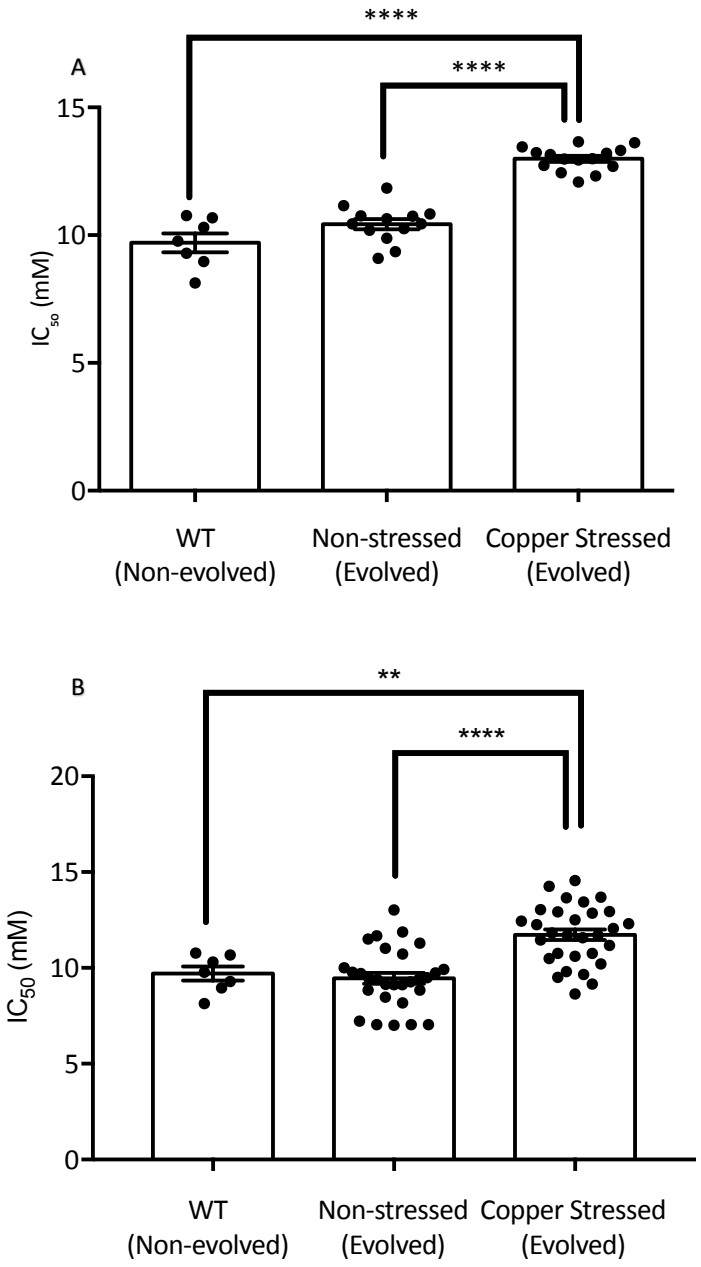

Figure *5*. Resistance to copper nitrate (mean resistance ± SEM) measured as IC_{50} derived from kill curve plots (examples shown later). **(A)** Mixed lineage resistance (WT n = 7, Non-stressed evolved n = 13, copper stress evolved n = 15) one way ANOVA, $F(2, 63) = 17.96$, $p < 0.0001$ **(B)** Single colony analysis (WT n = 7, Non-stressed evolved n = 29, copper stress evolved n = 30). * $p \leq 0.5$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

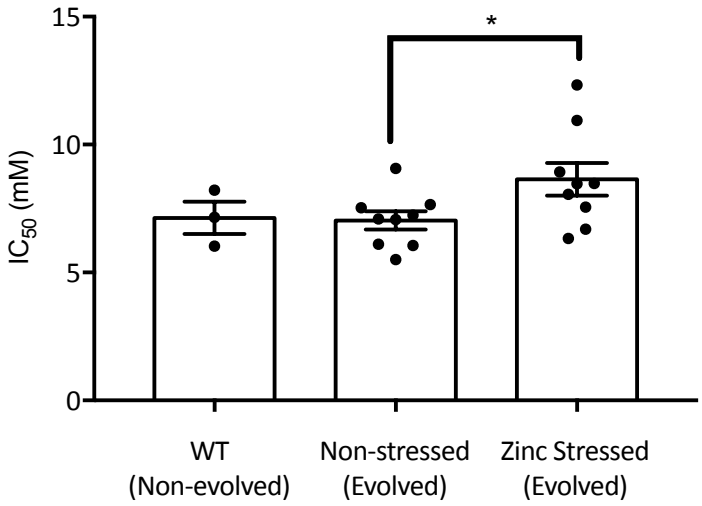

Figure 6. Resistance to zinc chloride (mean resistance ± SEM) measured as IC_{50} derived from kill curve plots. Single clone analysis only. 3 plots of each condition were produced per day, the averages were taken from the 9 days of experimentation, and these are plotted here (paired t-test, $t(8) = 2.786$, $p = 0.0237$) * $p \leq 0.5$.

INCREASED NON-GENOTYPIC HETEROGENEITY IS SUGGESTED TO EVOLVE IN RESPONSE TO COPPER NITRATE EXPOSURE, BUT NOT IN RESPONSE TO ZINC CHLORIDE EXPOSURE

Initially, mixed populations (i.e., aliquots from entire culture, rather than after individual-colony isolation) were assessed for heterogeneity and resistance in response to copper nitrate stress using the kill curve protocol. Heterogeneity was determined according to the gradients of the kill curves (Sumner *et al.*, 2003; Holland *et al.,* 2014).

The Hillslope parameter (generated with Prism software) reflects kill curve gradient. However, Hillslope values at the upper end of the range are very sensitive to small changes in the gradients of the steeper slopes. Due to this, the modulus of the Hillslope value was logged to give a dataset that would be less susceptible to skewing by large variation in the higher values. A Hillslope value close to 0 indicates a high level of heterogeneity (shallow kill-curve gradient), while a large value indicates low heterogeneity. Examples of curves produced from single colonies and their corresponding IC_{50} (mean resistance) and heterogeneity values can be seen in Table 4 and Figure 7.

When producing kill curves from mixed lineage backgrounds, heterogeneity appeared to have increased from a parental WT level (log +ve Hillslope, 2.0 ± 0.2) after evolution both with copper (1.3 ± 0.1) and without (1.1 ± 0.041) (Figure 8A) (one way ANOVA $F(2, 32) = 14.09$, $p < 0.0001$). When comparing individual clones, heterogeneity was not significantly different between the parental WT and clones evolved without copper (1.69 ± 0.106). However, both showed significantly lower heterogeneity than isolates evolved with copper stress (1.2 ± 0.032) (one way ANOVA $F(2, 63) = 10.39$, $p < 0.0001$) (Figure 8B).

However, though the increase in resistance remained stable after a further round of freezing (figure 9), the increase in NGH was not reproducible (figure 10).

Figure 11 shows the non-significant difference between the single isolates evolved with zinc, tested with zinc chloride kill curves, compared to either those evolved without stress and the parental WT.

Comparison between the heterogeneity and resistance (IC_{50}) of each individual isolate tested against copper nitrate (Figure 12A) suggested a weak negative correlation (r^2 = 0.200, p = 0.0002), while an even weaker relationship was observed with the separate isolates tested with zinc chloride (Figure 12B) (r^2 = 0.101, p = 0.161). This suggested that heterogeneity is not clearly related to mean culture resistance.

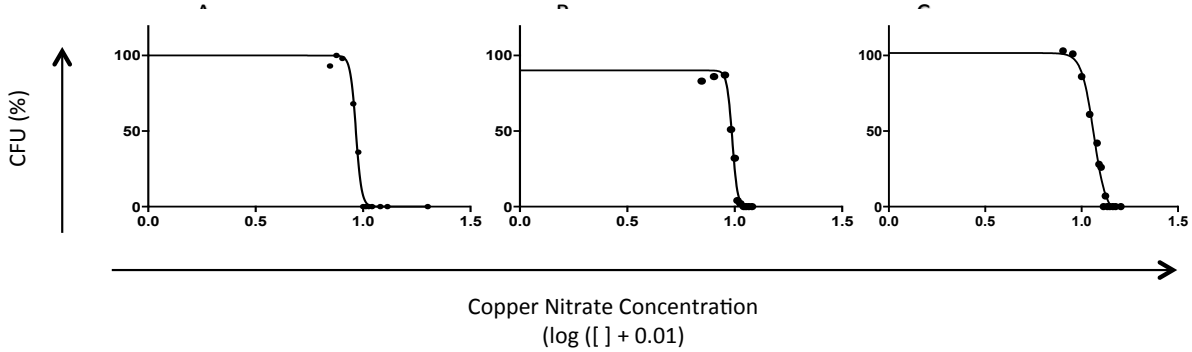

Figure 7. Percentage survival (CFU, colony forming units) compared to 0 mM copper nitrate is plotted against copper concentration for **(A)** WT, **(B)** an isolate evolved without copper nitrate, and **(C)** an isolate evolved with copper nitrate. Sigmoidal fitting is forced through 0% but not through 100% (Holland *et al.,* 2014). Example kill curve plots based on; Non-stressed evolved WT n = 7, n = 13, copper stress evolved n = 15.

Table 4. One set of representative example data from kill curve plots shown in Figure 3. Percentage survival compared to 0 mM copper nitrate is plotted against copper concentration for **(A)** WT, **(B)** an isolate evolved without copper nitrate, and **(C)** an isolate evolved with copper nitrate.

	A **WT** **(Non-evolved)**	**B** **Non-stressed** **(Evolved)**	**C** **Copper-stressed** **(Evolved)**
Resistance IC_{50} (mM)	9.29	9.64	11.49
Hillslope Kill curve Gradient	-36.87	-30.50	-15.84
Heterogeneity	Low	Low	High

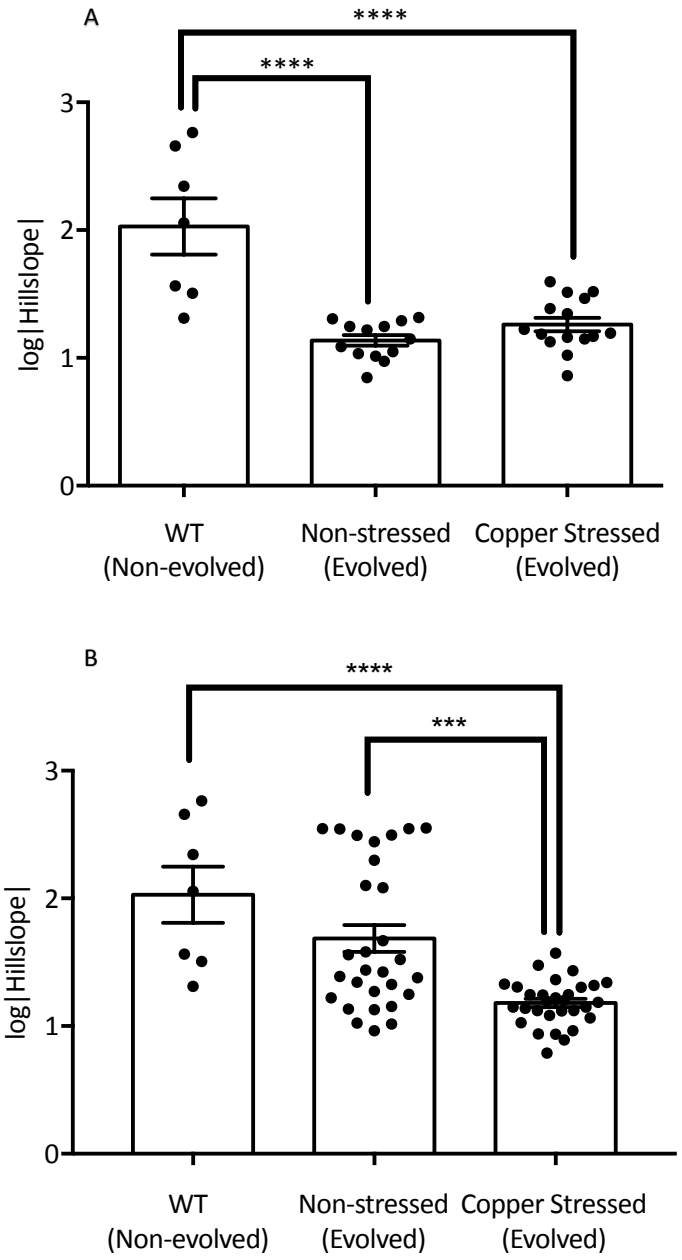

Figure 8. Heterogeneity measured as log|Hillslope| (mean ± SEM) derived from copper nitrate kill curve assay. **(A)** Mixed lineage analysis (WT n = 7, Non-stressed evolved n = 13, copper stress evolved n = 15) One way ANOVA $F(2, 32) = 14.09$, $p < 0.0001$. **(B)** Single clone analysis (parental WT n = 7, Non-stressed evolved n = 29, copper stress evolved n = 30). One way ANOVA $F(2, 63) = 10.39$, $p < 0.0001$. * $p \leq 0.5$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

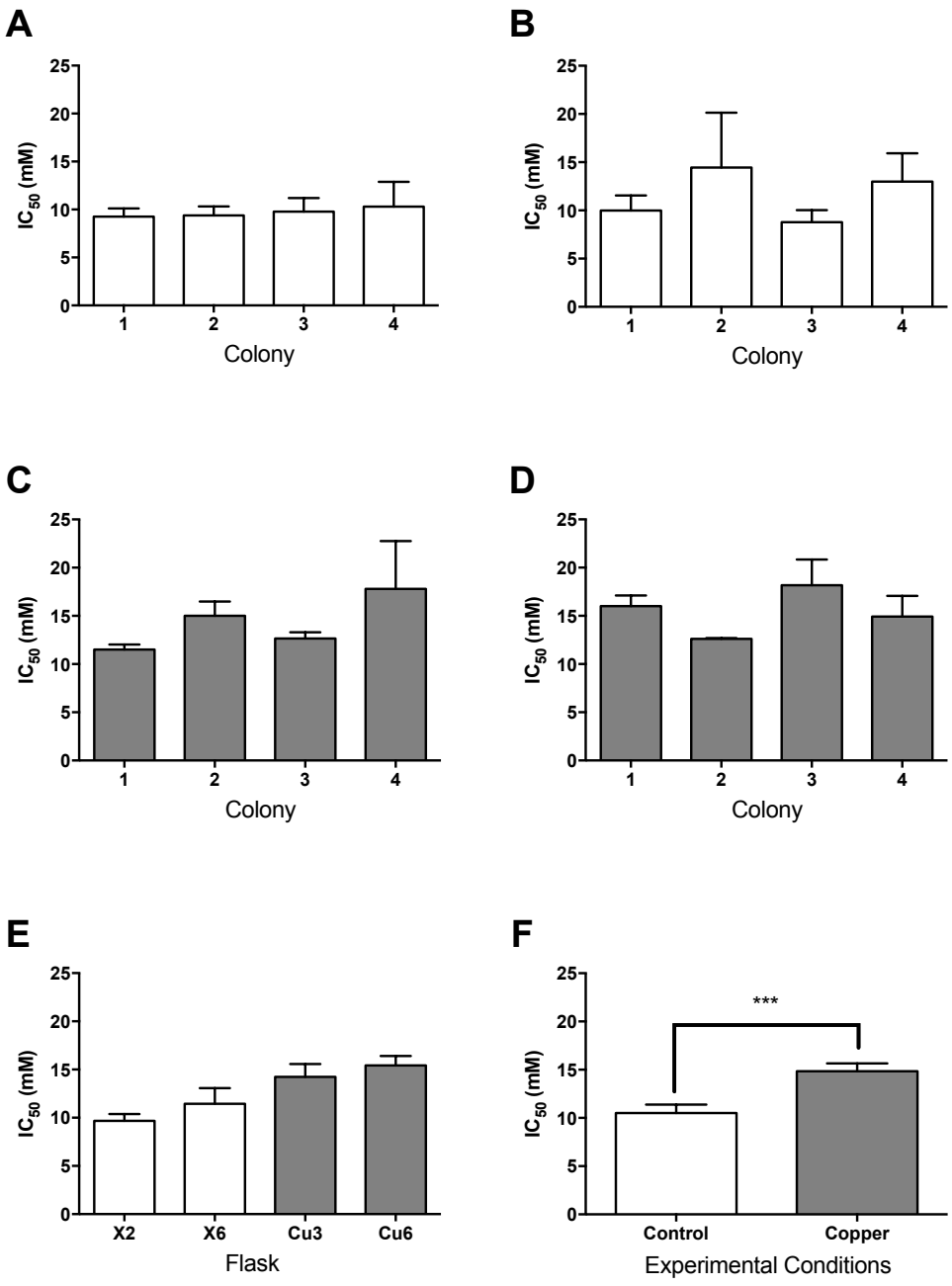

Figure 9. Resistance values derived from replicate kill curves of the same isolate. 4 isolates from flasks (A) X2 and (B) X6 (2 of the 10 parallel evolutions conducted under control conditions without stress) and 4 of the isolates from flasks (C) Cu3 and (D) Cu6 (2 of the 10 parallel evolutions conducted with copper nitrate stress) were repeated in triplicate. There were averaged within (E) their parallel evolution and (F) within evolutionary experimental conditions.

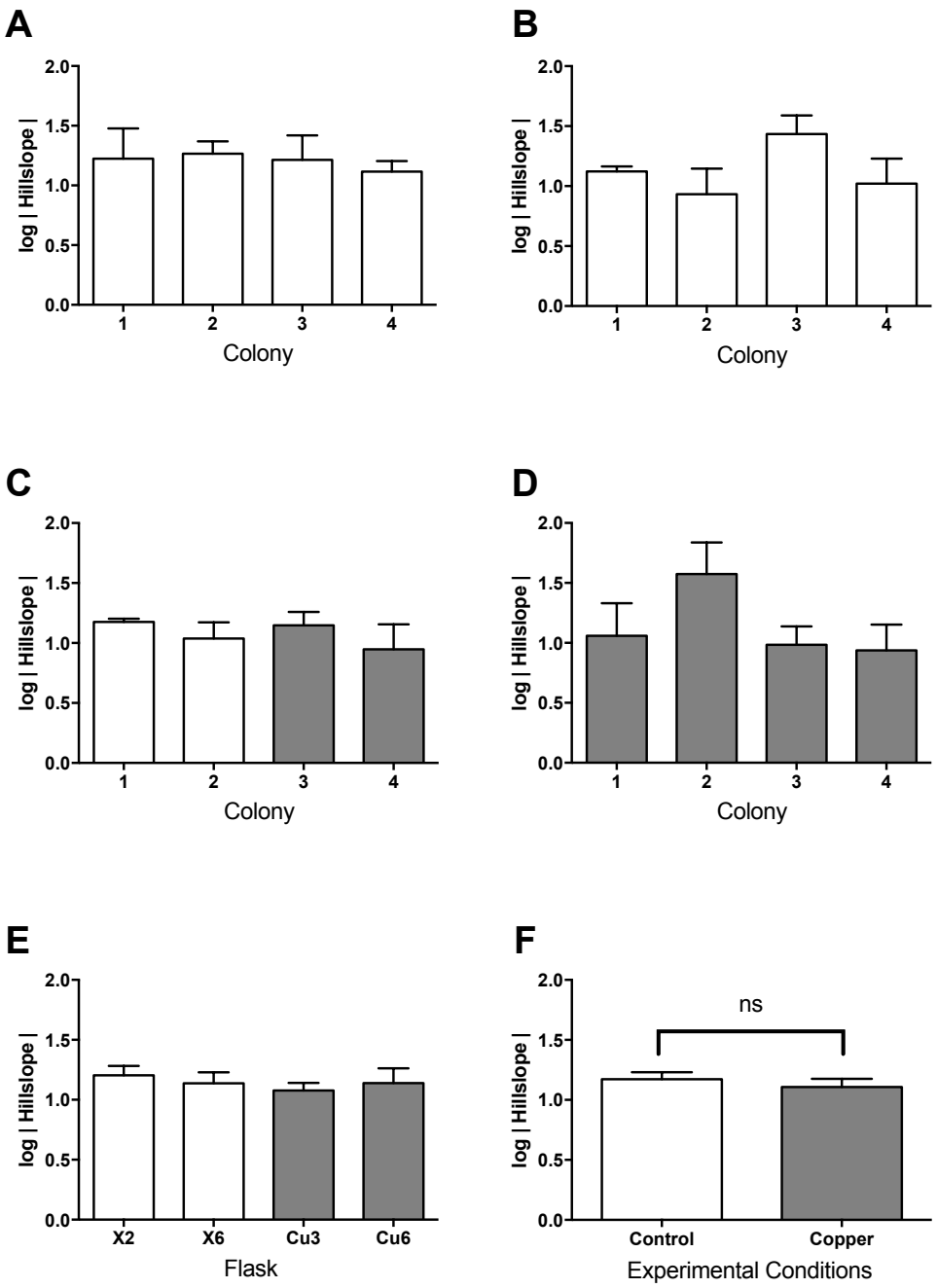

Figure 10. Hillslope (heterogeneity) values derived from replicate kill curves of the same isolate. 4 isolates from flasks (A) X2 and (B) X6 (2 of the 10 parallel evolutions conducted under control conditions without stress) and 4 of the isolates from flasks (C) Cu3 and (D) Cu6 (2 of the 10 parallel evolutions conducted with copper nitrate stress) were repeated in triplicate. There were averaged within (E) their parallel evolution and (F) within evolutionary experimental conditions.

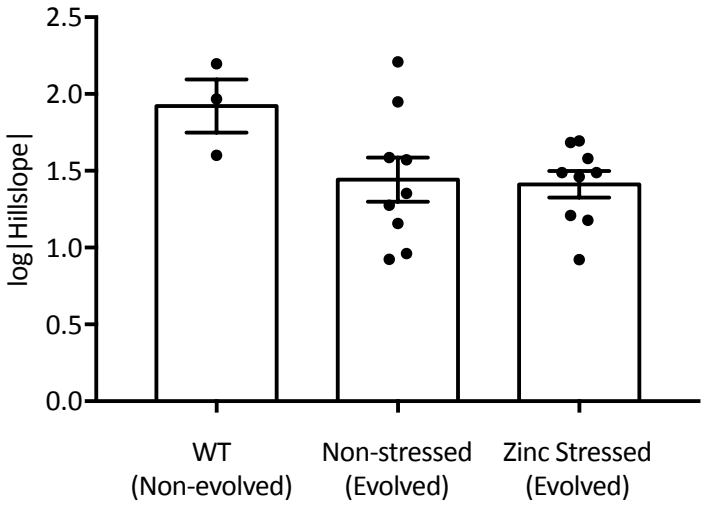

Figure 11. Heterogeneity to zinc chloride (mean resistance ± SEM) measured as log|Hillslope| derived from kill curve plots. Single clone analysis only. 3 plots of each condition were produced per day, the averages were taken for the 9 days of experimentation, and these are plotted here (paired t-test, $t(8) = 0.196$, $p = 0.850$). Non-significant.

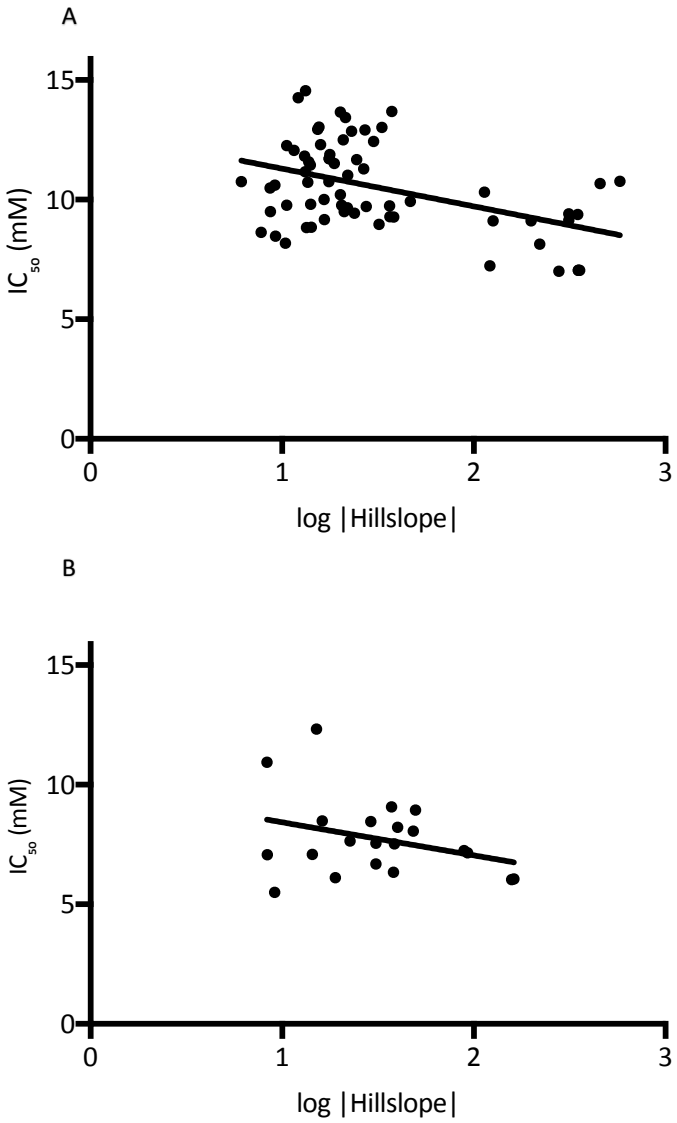

Figure 12. Correlation between heterogeneity (log|Hillslope|) and resistance (IC_{50}) for all isolates tested via kill curve assay with (A) copper nitrate (r^2 = 0.200, p = 0.0002) and (B) zinc chloride (r^2 = 0.101, p = 0.161).

FLOW CYTOMETRIC DETERMINATION OF VARIATION IN PHLOXINE B STAINING (CELL VITALITY) UNDER STRESS AND CELL SIZE VARIATION UNDER STRESS IN EVOLVED POPULATIONS

When populations of cells are exposed to increasing copper stress, variation in both cell size and Phloxine B staining (an indicator of cell vitality) (Noda, 2008) could be used to indicate non-genotypic heterogeneity. Isogenic populations of cells were exposed to increasing doses of copper nitrate to determine how this variation changes compared to a non-stressed culture (Figure 13). Populations were gated to exclude cells that fell in the region of a dead cell control (exposed to high heat), so that only viable cells were used in the analysis.

As cellular activity is inhibited, the cells are less able to pump the vitality stain out of the cell in an active manner. Therefore as concentration of copper nitrate increases, the median Phloxine B absorbance of cells increases. However, it is the range (diversity) of absorbance that would indicate the non-genotypic heterogeneity in cell vitality. The co-efficient of quartile deviation (CQD) values for Phloxine B absorbance, corrected for cell size (FSC), at each of the concentrations were used to create a standard curve for each strain. This could be used to compare heterogeneity at equivalent stress levels in different tests. The level of stress imposed on cells was estimated from the % increase in mean phloxine B fluorescence (% decrease in estimated vitality) at each dose. Doses giving an approximate 20% change were selected for heterogeneity comparisons. 4 isolates were tested per condition, though inability to calculate equivalent stress meant that not all replicates could be used in the final analysis. Cells that had been evolved with copper nitrate showed a non-significant increased range (heterogeneity) of phloxine B absorbances (26.78% ± 9.05) compared to parental WT (13.63% ± 6.43) and cells that had been evolved without copper nitrate (15.6% ± 2.11) (one way ANOVA $F(2, 6) = 1.367$, $p = 0.324$) (Figure 14A).

Forward scatter measurements with flow cytometry additionally provide information on cell size. This was capitalised on to examine for potential changes in cell size distributions (as an alternative heterogeneity phenotype) in the evolved populations. There appeared to be little effect on cell size variation (Figure 14B). Here, cells that had been evolved with copper nitrate showed a mean increase in cell size variation (3.94% ± 10.13) but that was mainly driven by a single outlying replicate, while WT (-8.35% ± 1.900) and cells that have been evolved without copper nitrate (-6.47% ± 3.766) show a mean decrease (one way ANOVA, $F(2,7) = 0.7377, p = 0.512$).

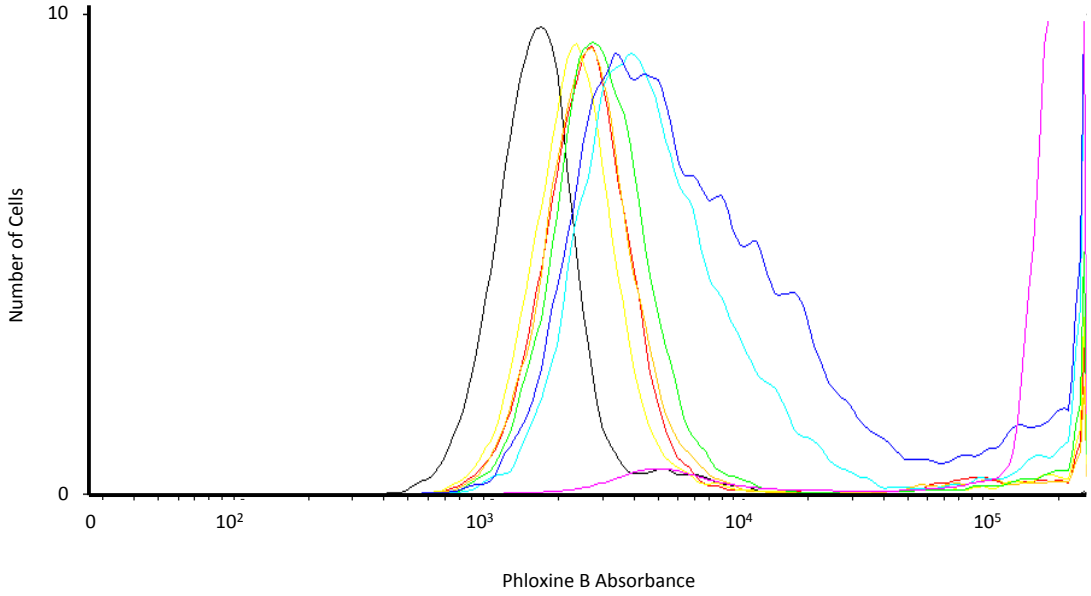

Figure 13. Example of Phloxine B absorbance profiles after exposure of yeast cells to a range of copper nitrate concentrations: controls with no dye (–), and heat killed with dye (–) displayed along with stained cells treated with copper nitrate concentrations of 0 mM (–), 1 mM (–), 2 mM (–), 4 mM (–), 6 mM (–), 8 mM (–).

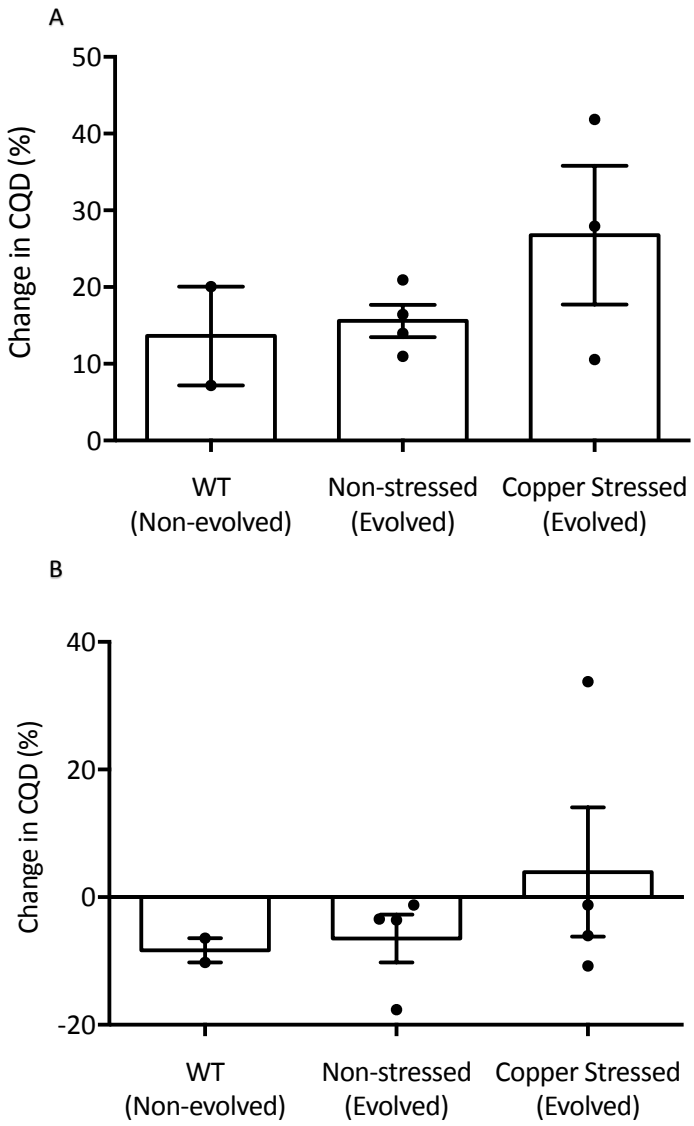

Figure 14. Percentage change in variation in **(A)** phloxine B absorbance (One way ANOVA F(2, 6) = 1.367, p = 0.324) and **(B)** cell size (one way ANOVA, F(2,7) = 0.7377,p = 0.512) in populations of parental WT cells, cells evolved without stress for ≥600 generations, and cells evolved with copper stress for ≥600 generations. A range of intensities of copper nitrate were tested to create a standard curve for each strain, then interpolated to read at 20% effect-size, in order to normalize for differences in resistance between isolates. * $p \leq 0.5$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

COLONY SIZE VARIATION INCREASES WHEN CELLS ARE EVOLVED WITH COPPER NITRATE FOR ≥ 600 GENERATIONS, BUT THIS CHANGE IS NOT INDEFINITELY STABLE

Cultures from single colony isolates were plated onto YEPD agar with no stress imposed (Figure 15A, B). Variation in colony size was evident from the range of colony sizes that were visible, in some cases reflected by an apparent bimodal distribution of large and small colonies (Figure 15C, D). 10 isolates from 3 parallel-evolved cultures (30 total per condition) were tested for colony size variation. Variation in colony size (measured as CV) was increased when populations were evolved with copper nitrate (0.349 ± 0.023) compared to when evolution took place with no added copper (0.249 ± 0.009) (unpaired t-test, $t(58) = 3.879$, $p = 0.0003$) (Figure 16A). Mean colony size after evolution with copper showed a decrease to 2.285 ± 0.182 mm from 3.668 ± 0.142 mm without copper (unpaired t-test, $t(58) = 5.985$, $p < 0.0001$) (Figure 16B). More WT repeats are necessary for reliable comparison between these and the other conditions.

When colonies that had previously been evolved with copper nitrate for a minimum of 600 generations were further batch cultured with no added copper (maintaining exponential phase as above), the colony size heterogeneity was stable for approximately 30 generations without copper but returned to a WT level after 60 generations (Figure 17). There was no significant difference in heterogeneity between the parental WT, isolates evolved without added copper for ≥600 generations, and previously-variable copper-evolved clones which had been evolved for a further 60 generations with no added copper (one way ANOVA, $F(2, 38) = 0.220$, $p = 0.803$). Each strain decreases from their original variation significantly once evolved for a further 60 generations without stress (paired t-test, $t(6) = 5.832$, $p = 0.001$). It was considered that the stability of the colony size heterogeneity phenotype for ≥30 generations suggested the likelihood that the phenotype may be due to evolved genotypic changes (which subsequently reverted) rather than

epigenetic or other effects that could be expected to revert over a shorter timescale (Avery, 2006).

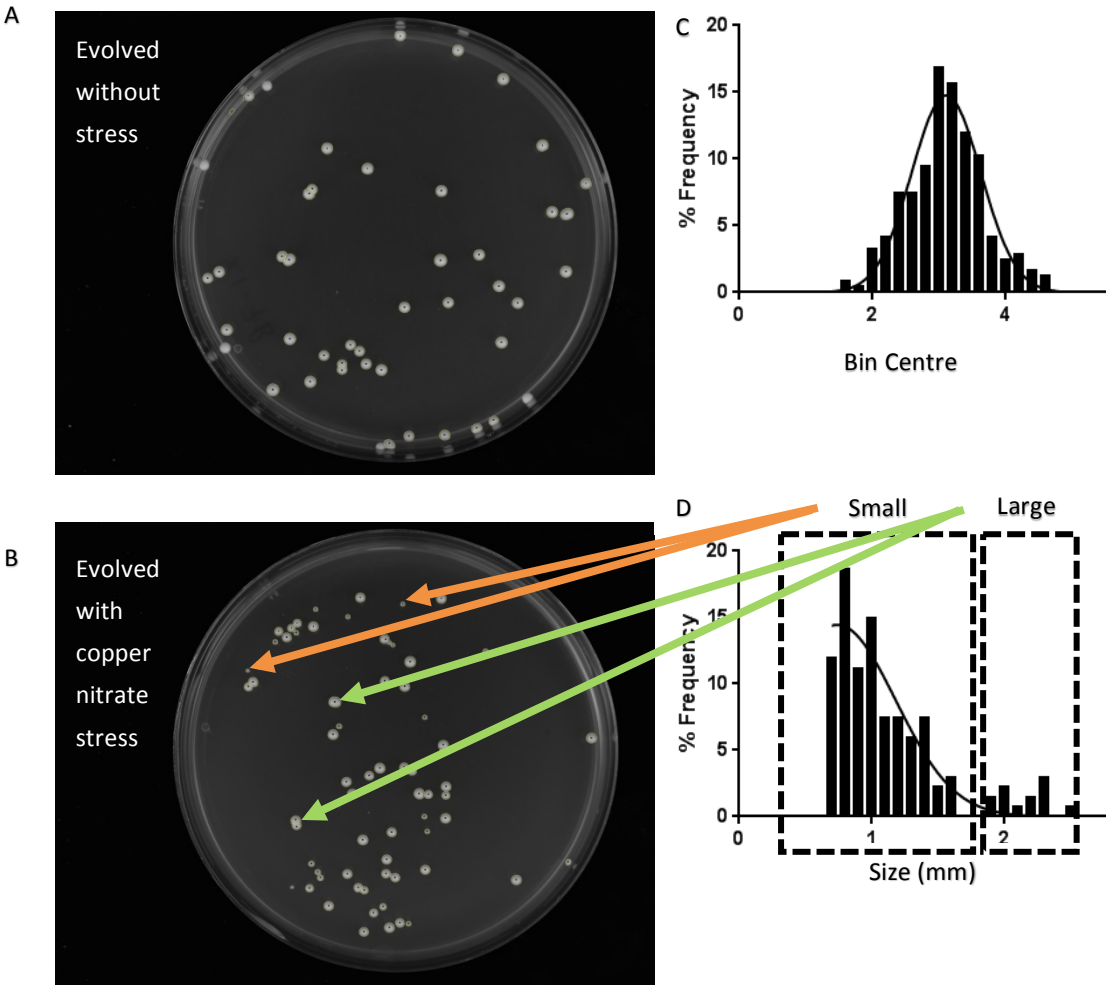

Figure 15. Scans of example colony size and variation on YEPD agar, and normality distributions for isolates evolved **(A, C)** without copper and **(B, D)** with copper. Normality distribution bars represent data intervals ranging 0.1 mm.

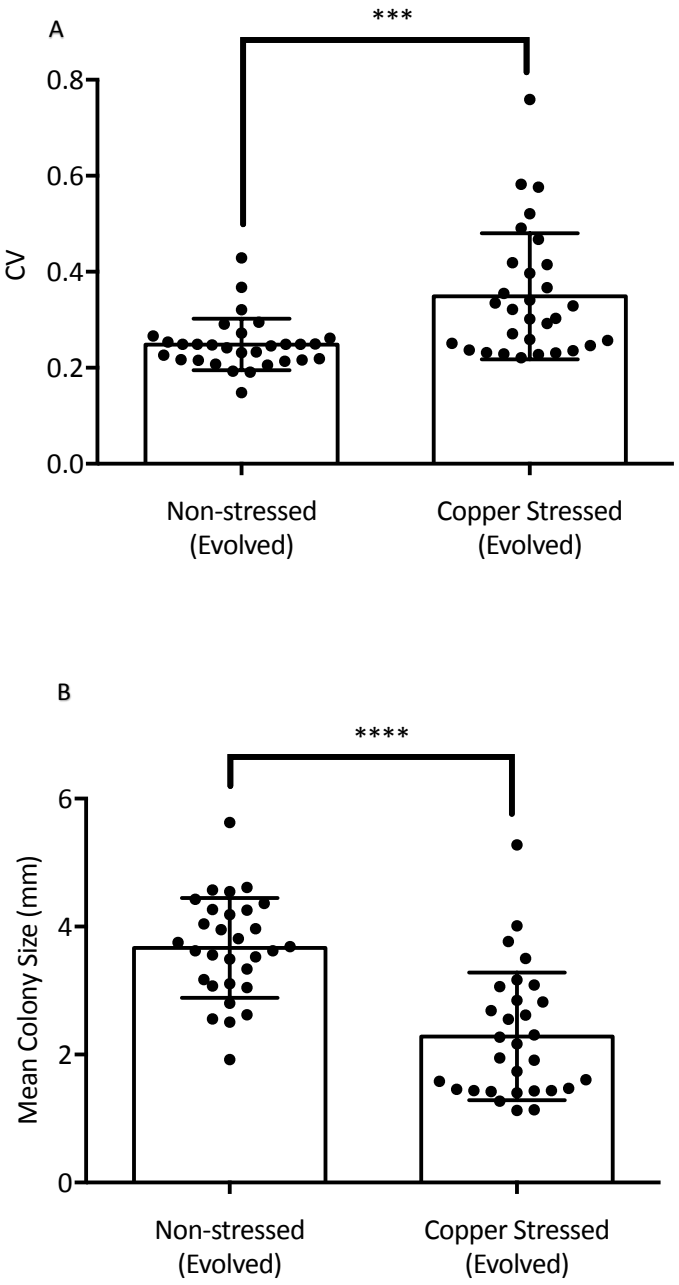

Figure 16. Colony size analysis on standard YEPD agar. 10 isolates from 3 parallel-evolved cultures (30 total per condition) were tested. **(A)** Colony size variation (mean ± SEM) measured as CV (unpaired t-test, $t(58) = 3.879$, $p = 0.0003$) and **(B)** mean size (mean ± SEM) (unpaired t-test, $t(58) = 5.985$, $p < 0.0001$). * $p \leq 0.5$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

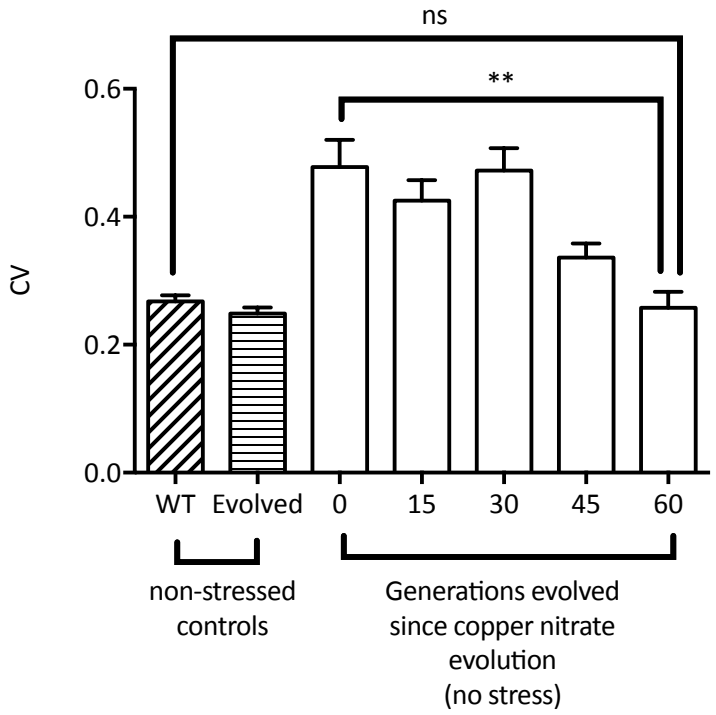

Figure 17. Colony size analysis on standard YEPD agar. Colony size variation (mean ± SEM) measured as CV for non-evolved WT, evolved without added copper (≥600 generations), and average CV across 9 clonal isolates previously evolved with copper nitrate (≥600 generations) and then subsequently for a minimum of 60 further generations with no added copper. ns $p > 0.05$, ** $p \leq 0.01$.

SMALL COLONIES MAY PROVIDE A FITNESS BENEFIT TO THE POPULATION AS A WHOLE

It was hypothesized that the colony size variation may reflect an adaptive evolutionary response to copper selection. First, to test the possibility that the smaller colonies that were evident may be petite (mitochondria-defective) mutants, colonies were restreaked onto fresh agar. Each restreak in turn produced a mix of small and large colonies (not shown), indicating that while the colony-size heterogeneity phenotype was heritable (above), the sizes of individuals within a clonal population were not. This indication that small colonies were not petites was supported with a TTC assay to test for respiration. All colonies showed red colouration, indicating that they were respiring. Petite mutants cannot respire so would remain white. Together, the results indicated that small colonies were not petites.

To test the hypothesis that the heterogeneity phenotype may relate to copper resistance, copper-evolved small or large colonies (selected by eye), or parental-WT colonies were suspended at equal cell densities in PBS, treated with 0, 5 or 10 mM copper nitrate for 12 hours with shaking (120 rpm, 24°C) then plated onto YEPD agar to assay relative survival (Figure 18). PBS was used to maintain viability but prevent growth. Viable cell numbers under control (minus copper) conditions were stable across the time course, facilitating quantification of copper-specific effects. Survival was assessed by comparison with the CFU determination at 0 hours. Under no stress, all three colony types remained at around 100% viability throughout. Under 10 mM copper, all three colony types showed loss of viability, with survival decreased to around 50-60% after 6 hours. However, under 5 mM stress, survival decreased to 70.0% ± 1.2 for small copper-evolved colonies and 58.3% ± 0.9 for parental-WT, but remained high at around 104.0% ± 4.6 after 6 hours for large copper-evolved colonies. In all cases, survival dropped to around 60-70% after 12 hours at 5 mM copper. This indicates that small copper-evolved colonies have a similar copper resistance to the parental-WT, while decreased copper resistance in the large colonies is detectable at least during short term

exposure to an intermediate copper concentration. This supports the hypothesis that the variable colony-size phenotype may impact copper resistance of copper-evolved cultures.

Colonies are visibly darker upon growth with copper nitrate on YNB agar (Figure 19). Colony colour change indicates degree of copper uptake (Yu, *et al.*, 1996). This is the case both for cells evolved with copper nitrate previously, and those that were not. The copper-evolved isolates show higher copper resistance on agar, reflected by colony formation at higher dilutions of spotted cell suspension. However, these copper-evolved colonies are darker in colour, suggesting that they may be accumulating at least as much copper as the cultures evolved without copper. This might indicate that these strains are able to uptake more copper before toxic effects are observed.

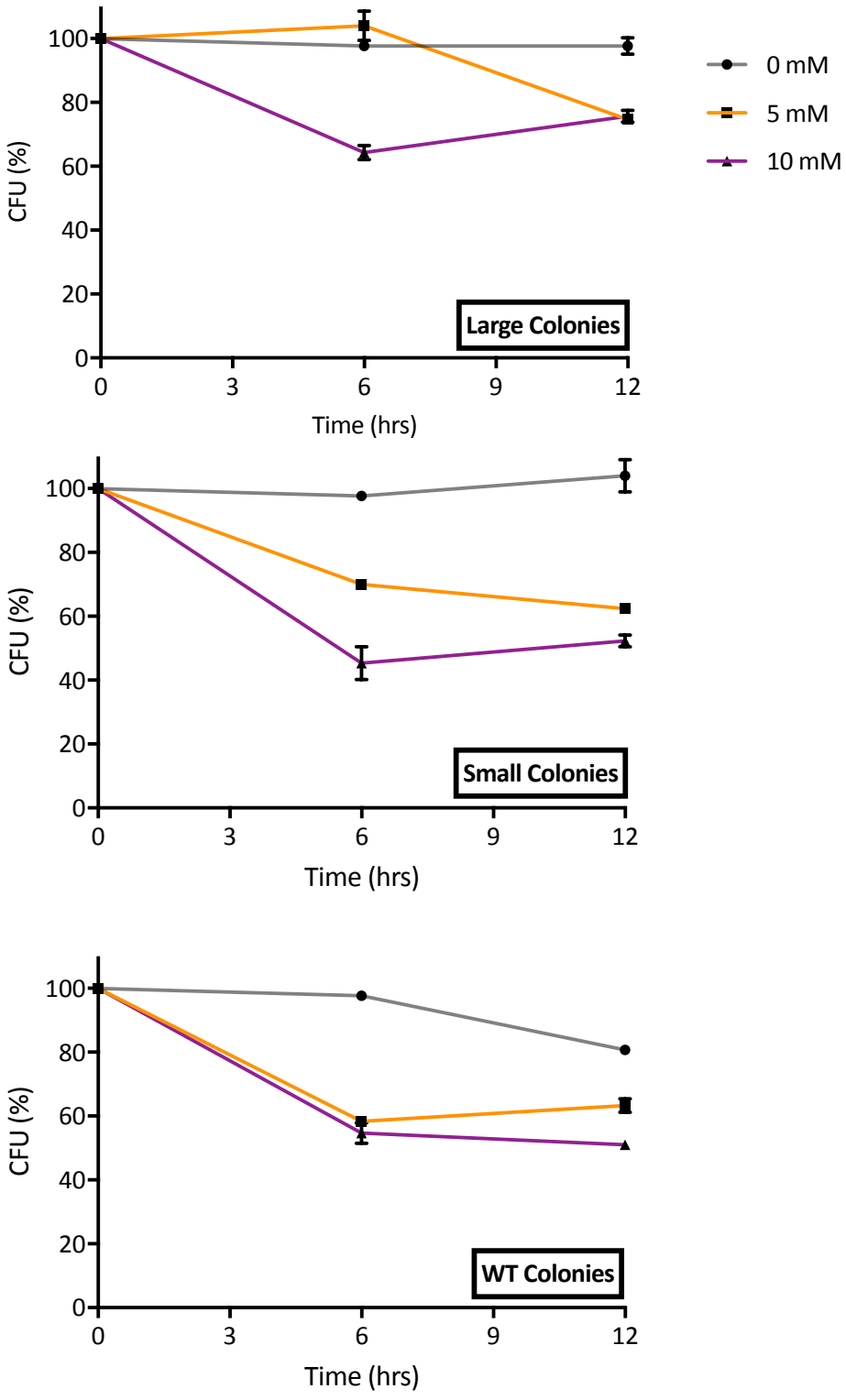

Figure 18. Percentage survival (mean ± SEM) compared to 0 hrs for large copper-evolved, small copper-evolved and parental-WT colonies exposed to 0, 5 or 10 mM copper nitrate in PBS over a 12 hours time period. Mean of biological triplicates of Cu3-1 shown.

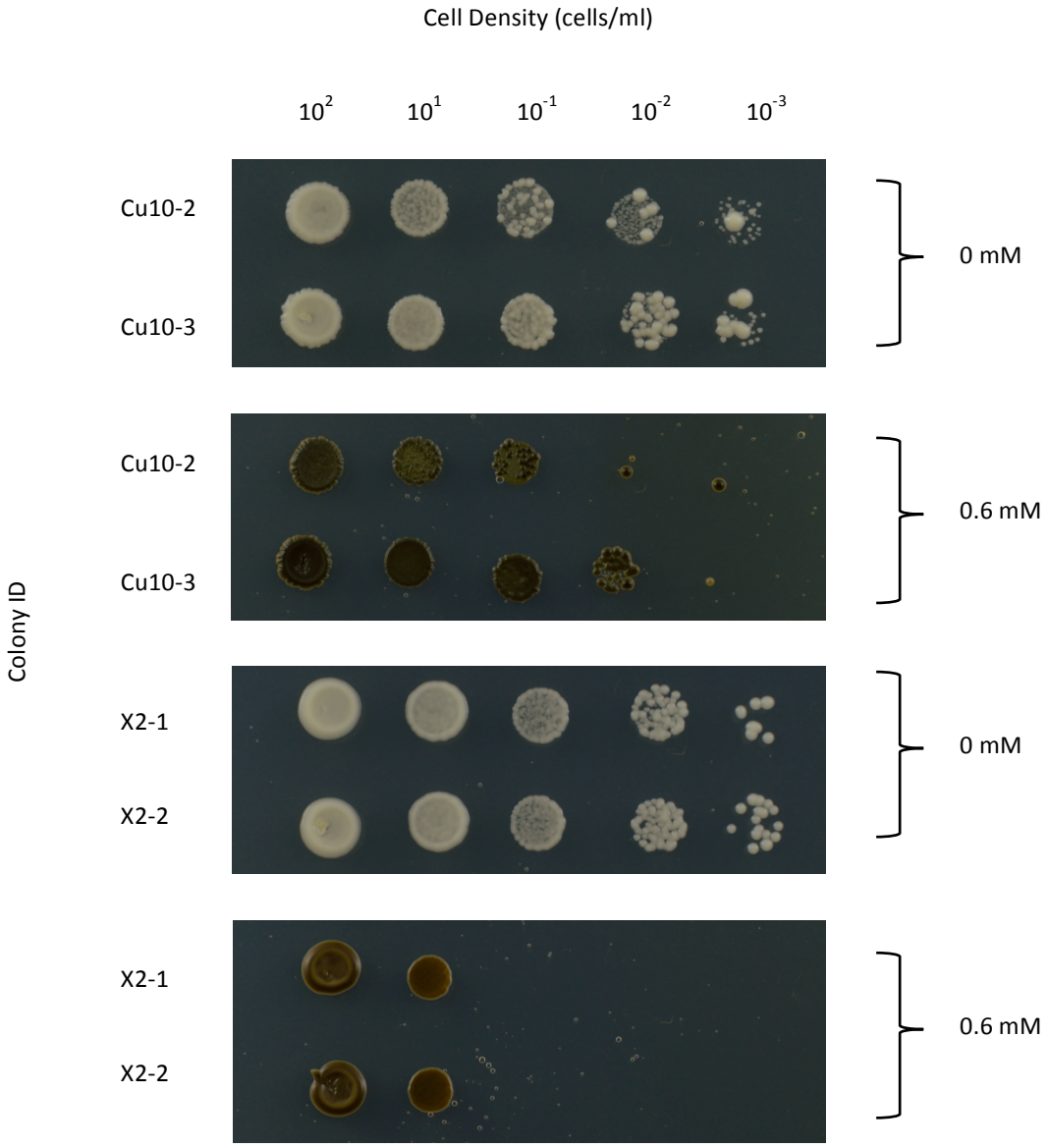

Figure 19. Spotting on YNB with copper nitrate for 2 isolates evolved from with copper nitrate (Cu10-2 and Cu10-3) and two isolates evolved without added copper (X2-1 and X2-2). The 2 isolates were from the same parallel evolution. Cultures were spotted on to YNB agar supplemented with 0 mM or 0.6 mM copper nitrate.

SEQUENCING

Due to the maintenance of the colony size variation for 30 generations, it is likely that the change is genetic. Full genome sequencing was anticipated to identify any SNP changes. Though many changes are likely to be unrelated to the observed changes in phenotype, the SNP(s) that are correlated with the phenotypes will be among those identified.

A total of 23 strains were selected to send for sequencing as shown in Table 5, and determined by values shown in Appendix I. Of the 10 parallel 600 generation evolutions conducted under each condition, 3 had been tested for heterogeneity and resistance phenotypes. From these 3 lines therefore, 3 isolates were selected each, along with 4 representative isolates from lines evolved without copper, and the WT strain. In addition to this, isolates that were chosen corresponded to those that had been further evolved without copper stress for 60 generations.

Once DNA was extracted, presence of DNA was visually assessed using agarose gel visualisation (Figure 20). Bands were present for all 23 samples, though fainter for samples 1, 2 and 5. Quality was then assessed by collecting nanodrop ratios (Table 6). 260/280 ratio indicates the purity of the DNA. A low ratio may indicate the presence of ethanol remaining from the extraction process. This can interfere with upstream processes and must be avoided. Ratios are high, so ethanol contamination is not likely to be an issue with these samples. The 260/230 ratio is a secondary measure of purity, and are expected to be around 2.0 - 2.2. Low ratios may indicate the presence of phenol, carbohydrates or EDTA buffer. Again, ratios do not indicate that this will be an issue.

Unfortunately the sequencing is still in progress and results will therefore fall outside of the scope of this report.

C. ELEGANS

Once sequencing results reveal which genes show SNPs within regulatory regions, homologues in higher organisms might then be identified. One such opportunity to extend our knowledge will be by using the experimentally evolved *C. elegans,* evolved here with copper and zinc. Each 10 parallel evolutionary lines were frozen to preserve any genetic changes which have taken place throughout the evolution. However, time did not allow for any further genetic analysis in the present study.

Taking this another step further, comparisons could be made to larger multi-cellular organisms. The stickleback fish *Gasterosteus acueatus* has been identified to show variation in populations affected by copper pollution and offers a great opportunity to study individual cell variation in larger scale organisms in the wild.

Table 5. Sample ID and corresponding strain ID. BY4741 is the haploid *Saccharomyces cerevisiae*, 2-5 are strains that were evolved without stress (indicated as X), 6-14 are strains that were evolved with copper stress (indicated by Cu), 15-23 correspond to the same strains as Cu, but with a further 60 generations evolution with no stress. Subsequent numbers refer to evolution flask number, then isolate number. The values represent a single isolate replicate from parallel evolutions.

SAMPLE #	STRAIN ID	Kill Curves		Colony Size Variation	
		log|Hillslope|	IC50	Mean	CV
NON-EVOLVED – ANCESTRAL WILD.TYPE					
1	BY4741				
EVOLVED – 600 GENS WITH NO STRESS					
2	X1-1	2.543	9.384	1.923	0.246
3	X1-3	2.495	9.134	3.110	0.216
4	X1-5	2.101	9.119	3.074	0.266
5	X2-7	2.496	9.401	3.950	0.248
EVOLVED – 600 GENS WITH COPPER STRESS					
6	Cu3-1 A	1.244	10.760	1.400	0.468
7	Cu3-5 A	1.341	9.658	1.609	0.329
8	Cu3-6 A	1.149	9.810	1.911	0.355
9	Cu6-1 A	1.572	13.690	1.455	0.582
10	Cu6-9 A	0.963	10.610	2.849	0.491
11	Cu6-10 A	0.890	8.634	3.503	0.521
12	Cu10-1 A	1.084	14.260	1.439	0.759
13	Cu10-3 A	1.330	13.440	1.434	0.576
14	Cu10-9 A	1.121	11.170	5.278	0.397
EVOLVED – 600 GENS WITH COPPER STRESS + 60 GENS WITH NO STRESS					
15	Cu3-1 B				
16	Cu3-5 B				
17	Cu3-6 B				
18	Cu6-1 B				
19	Cu6-9 B				
20	Cu6-10 B				
21	Cu10-1 B				
22	Cu10-3 B				
23	Cu10-9 B				

Table 6. Qubit concentration and Nanodrop quality values corresponding to strains identified in Table 5.

Sample name	Qubit concentration ng/ul	Nanodrop ratio A260/A280	Nanodrop ratio A260/A230
1	70	2.111	1.797
2	90	2.102	2.023
3	110	2.106	2.166
4	97.5	2.112	2.202
5	100	2.129	2.007
6	113	2.098	2.154
7	97.5	2.109	2.172
8	110	2.108	2.178
9	92.5	2.107	2.263
10	92.5	2.132	2.212
11	90	2.126	2.155
12	95	2.099	2.21
13	92.5	2.076	2.159
14	92.5	2.082	2.211
15	125	2.082	2.216
16	118	2.101	2.222
17	105	2.075	2.215
18	97.5	2.086	2.203
19	95	2.126	2.231
20	97.5	2.075	2.164
21	120	2.106	2.066
22	108	2.073	2.172
23	102	2.088	2.233

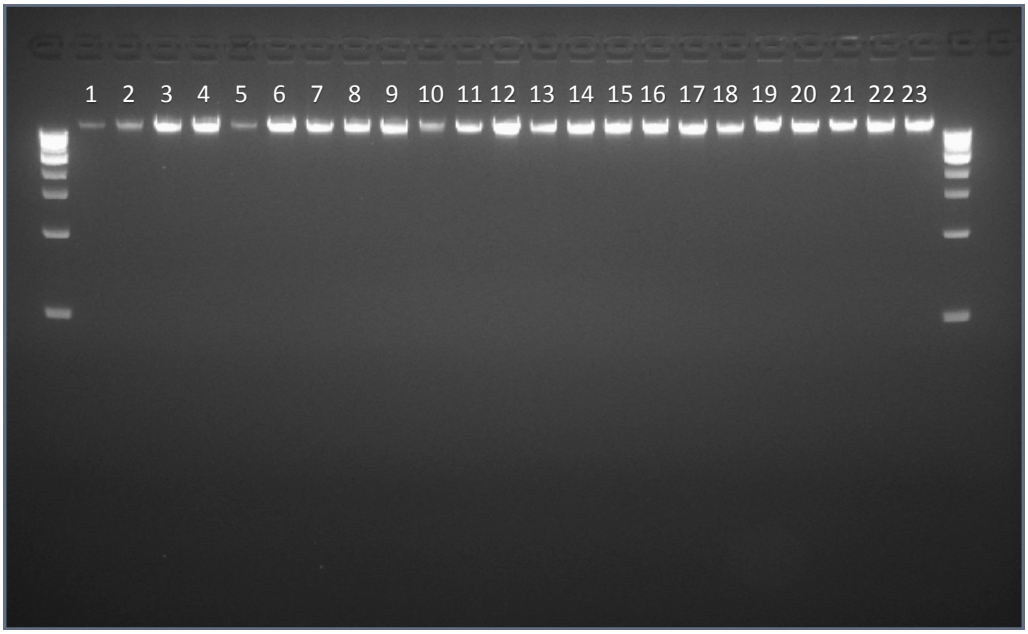

Figure 20. 5 ul DNA samples 1-23 (as described in Table 5) loaded onto 2 % agarose gel. Run at 100 V for 60 mins. Displayed with 1 kb DNA ladder (NEB #N3232) in the bordering wells.

DISCUSSION

In this study, we have observed an increase in resistance to heavy metals in the haploid strain of the well-studied yeast, *Saccharomyces cerevisiae,* after prolonged growth in media supplemented with two such metals. Populations evolved for a minimum of 600 generations with either copper nitrate or zinc chloride demonstrated a significant increase in resistance specific to the metal with which they were evolved compared to cells evolved in equivalent conditions without metal stress. Cells in stationary phase are relatively dormant. When cells from a culture in stationary phase are exposed to a new stressful environment, they will delay the first division (Fridman, *et al*., 2014). Care was taken here to reduce the risk of cultures entering stationary phase throughout the duration of the evolution by experimenting with different growth conditions (media type and temperature) and level of stress (concentration of the metal in question) to reduce doubling time.

Metal resistance was confirmed through IC_{50} values derived from kill curve plots. As measurement of heterogeneity using kill curves is a relatively new advancement in microbial science, the inclusion of a positive control is needed to validate conclusions drawn from this methodology. Since increased resistance to high levels of heavy metals in a range of organisms has been well documented previously including in microbes (Nies, 1999), the confirmation of this increase in resistance can act as the positive control.

All evolved populations originated from a single clonal population. Aliquots of the culture at the final evolutionary time point were frozen to preserve a representation of the mixed lineage evolutionary outcome. All tests presented here were conducted using cultures originating from a single colony and allowed to replicate for no more than 16 generations.

It has been widely suggested that increased NGH gives populations an advantage in the face of environmental uncertainty (Acar, *et al.,* 2008; Levy & Siegal, 2008; Levy, *et al.*, 2015). Here, increased NGH was suggested in

isolates from populations evolved whilst exposed to copper, observed both from a shallower kill curve gradient and in a non-significant increase in Coefficient of Variation (CV) of Phloxine B absorbance compared to cells evolved without copper. This supports previous findings in the host lab suggesting that NGH is widespread in wild yeast populations, and that high levels of pollution in natural environments leads to high levels of NGH in multiple wild yeast strains. In this previous study, isolates of *C. sake* which had been identified to have with high NGH displayed increased levels of fitness than isolates with lower heterogeneity when exposed to lead, which they had previously been exposed to in the wild, which again has been complemented here.

Many species, including yeast, use copper as an important catalytic co-factor. It is used in many important pathways such as oxidative defence, respiration and iron transport (Graden & Wings, 1997) which are essential for normal development. However, when it is accumulated above the metabolic requirement of the cell, it can be highly toxic, as describe by Peña *et al.* (1999). It not only displaces other metal co-factors (disrupting essential metabolic processes), but also produces damaging ROS. Cells therefore require a delicate system for navigating this fine threshold between the two states. It is for this reason that noise around genes responsible for maintaining this balance is limited, resulting in a low natural level of NGH as observed by Holland *et al.* (2014) in wild yeast populations exposed to low levels of copper stress, and within the WT *S. cerevisiae* BY4741 populations tested here. When exposed to long-term copper stress, there may be ample opportunity to evolve increased efficiency in positive feedback loops involved in this biological balancing-act. This might explain the observed increase in NGH seen here after controlled evolution, and in wild isolates collected besides a the copper-polluted lake near the Devon Great Consols mine complex, in the UK (Holland *et al.*, 2014).

However, this increase did not appear to be stable. After a further found of freezing this difference could no longer be identified. The increase in NGH

indicates the presence of rare persister cells. These cells may be less fit under standard conditions (Levy, *et al.,* 2012). They benefit the population as a whole by being able to survive higher levels of stress than the average cells in the population. According to Hamilton's rule (1964), altruistic behaviours can spread through a population if the cost to the individual is less than the benefit to a relative. As genes are shared between closely related individuals, if an individual expresses a behaviour that doesn't enable it to reproduce itself, but enables others in the population to reproduce, some of its genes are still passed on to the next generation. This might go some way to explaining why some individuals express costly behaviours for the benefit of the population as a whole. However, as NGH appears very costly, it is likely to be lost quickly from the population once removed from the stressful evolutionary conditions. A representative schematic of the path that cultures took once removed from the evolutionary conditions is shown in figure 21. For ease of argument, we will say that each time an over night culture is prepared, or an agar plate inoculated, the culture grows for 10 generations. The initial round of kill curves would have undergone 20 generations of growth, and a single cell bottleneck. The second round of kill curves tested as replicates were conducted on cells that had undergone a further 20 generations of growth, and a second bottleneck. If the phenotype is lost quickly, is a further 20 generations enough to lose it?

No increase in NGH was observed after exposure to zinc. It has previously been shown that copper can act as a mutagen, both with direct exposure to DNA (Tkeshelashvili, *et al.,* 1991), and *in vivo* in mouse tissue (Pra, *et al.,* 2008). It is possible that resistance evolves quickly, while evolution of NGH takes a little longer. During the timescale of the evolution shown here, resistance would naturally evolve early on. If copper is acting as a mutagen, it may in fact have increased the evolutionary pressure on those cells evolved with copper, compared to those evolved with zinc, encouraging the mutation that would allow an increase in NGH. Alternatively, as WT *S. cerevisiae* BY4741 populations exposed to zinc already show a larger degree of NGH than when

exposed to copper, the genome may have already reached an evolutionary maximum (Silander, *et al.,* 2007). It is evident from a plethora of evolutionary studies that fitness cannot increase indefinitely, but will eventually reach a plateau.

Also observed was an increased NGH in the form of colony size variation. Isolates evolved with copper showed increased variation compared to both the WT and isolates evolved without stress. This increase in variation was visible not only when re-exposed to copper, but even when the cells were not exposed to stress. This colony size variation could indicate cellular co-operation. There are two clear sub-populations, identifiable on agar as large and small colonies. We could explain this using the Continuous Prisoner's Dilemma in game theory.

Continuous Prisoner's Dilemma is a game incorporating two strategies; co-operate or defect. We must consider the investment required by the individual. An individual is more likely to co-operate if the cost to the self is small, but the benefit to the other is large (Killingback, *et al.,* 1999). Preliminary results indicated that the large colony sub-population is more fit upon exposure to copper than either the small colony sub-population, or WT colonies. It is possible that the evolutionary culture formed two distinct populations, each expressing a costly phenotype for the benefit of the whole. The presence of small colonies under standard conditions would indicate that some cells have slower growth that is costly under normal conditions. The benefit of the small colonies is unclear here and requires further experimentation to uncover the advantage they give to the population. Co-operation has been found in a range of cellular populations, including in cancerous tumours (Axelrod, *et al.,* 2006) where neighbouring cancer cells were found to protect each other from host defences where alone they would not survive.

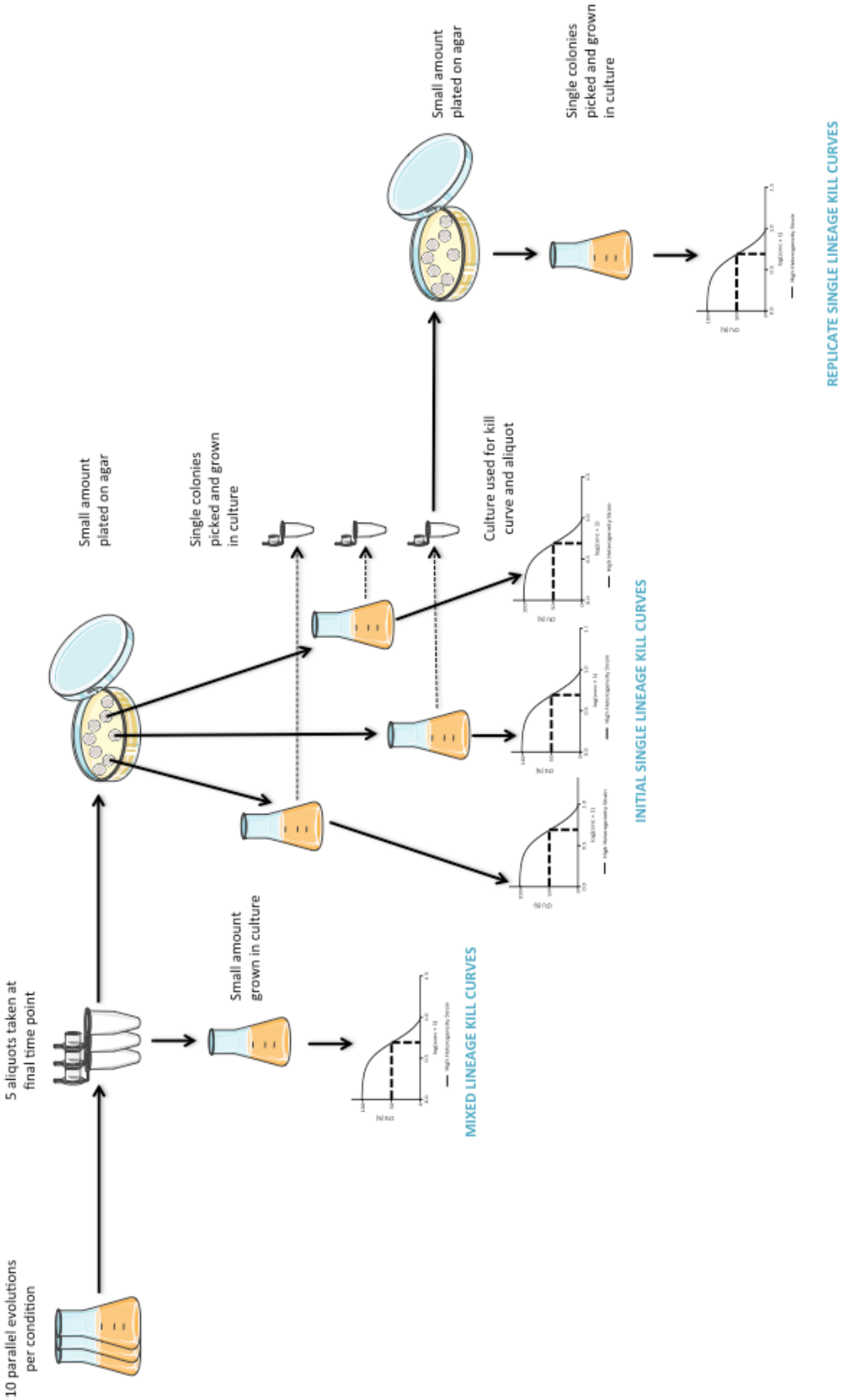

Figure 21. Schematic showing the path that cells took between evolutionary growth and experimental testing.

As the two sub-populations were no longer seen after 30 generations, this would indicate that the evolved phenotype is indeed costly to maintain once the stressful conditions have been removed. This finding complements the non-repeatability found during phenotype testing using the kill curve methodology, where after 20 generations removed from the stressful evolutionary conditions there was a significant difference between isolates evolved with copper and isolates evolved without copper, but the difference was no longer significant after 40 generations. Examine how long it takes for the phenotype to appear once under copper stress once more, relative to cells that have never before been exposed would add valuable insight. If cells previously exposed evolved the response more quickly, it would indicate cellular memory (Acar, *et al.,* 2008). In Holland et al. (2014) wild isolates of *C. sake* were evolved under controlled evolutionary conditions with the metal that they had previously been exposed to, and demonstrated that NGH could be selected for during long-term environmental stress. This may, again, be an indication of cellular memory.

It is important for individuals to be able alter their gene expression throughout their lifetime to match the environmental conditions they are encountering. Employing epigenetic changes allows such flexibility. Sometimes, these changes are also observed in the following generations (Daxinger & Whitelaw, 2010). Trans-generational epigenetic inheritance can occur where clearing of epigenetic markers within the genome, which usually takes place between generations, is incomplete. The mechanisms used to circumvent the normal genome clearing are poorly understood, so the potential for epigenetic markers to still be observed after 30 generations is uncertain. Genetic mutations can be lost quickly if the locus is under high selective pressure. If the phenotype is 'simple' and only requires mutations in a single gene, the phenotype may be lost after only a few generations (Rando & Verstrepen, 2007). NGH is unlikely to be a simple phenotype, increasing the likelihood that the change leading to the observed changes in phenotype here are due to a sequence-based genetic mutation. Genetic sequencing is

therefore likely to shed some light on the underlying causes. There are multiple genes already identified as important in stress response to copper and zinc, some of which are outlined below. We need to examine the regulatory regions of these genes.

Differential uptake of copper within individual cells in the population could explain an increase in NGH. Bicinchoninic acid (BCA) binds in a highly specific manner with copper (I), forming an intense purple complex. BCE could therefore by used to test for intercellular copper presence (Brenner & Harris, 1995). In possible future work, methodology could be adapted to allow the lysis of individual cells to determine variation in copper uptake. For example, when looking at colony size variation, does the small or large sub-population uptake more copper? Recent developments in flow cytometric methods have allowed the quantification of the uptake of stressors (such as metals) into individual cells (Crawford, *et al.*, 2016). Each cell passes an X-ray beam and total metal content can be quantified from μM to mM levels, though the ability to identify individual metals is still lacking.

There are many difficulties associated with studying single cell behaviour. Expression noise means that individual cells are somewhat unpredictable. That being said, for essential processes statistics dictate that the population is likely to tend towards a predictable average. For example, the fate of a coin flip is unpredictable, but when 100 coins are flipped it will result in an approximately equal mix of heads and tails. From a developmental point of view, this can be observed in *Drosophila* cell differentiation (Wernet & Desplan, 2004). Each photoreceptor cell has an uncertain path prior to patterning, but always results in a 70:30 ratio of pale to yellow ommatidia. If we look at figure 8A compared to figure 8B, we would miss a lot of the story if we simply looked at the average. On average there appears to be no difference, but looking at the single isolate level, we can see that there is a wide variety of behaviours at the individual cell level that is contributing to this average.

During evolution, many lineages will compete, and purely due to chance, many successful mutations will not increase beyond extremely low frequencies. Recent advances in technology have allowed lineages to be tracked throughout an experimental evolution (Levy, *et al.*, 2015). A library of around 500,000 'barcodes' (random 20 bp sequences) were used to integrate traceable elements into the genome in *S. cerevisiae.* As previously discovered, final evolutionary outcomes can be striking different, despite starting from a single parental genotype (Lenski, *et al.*, 1991; Vasi, *et al.,* 1994). Though this was reiterated in this study, tracking initial competition showed that early evolution followed predictable patterns where small effect mutations were eventually outcompeted by rare large effect mutations. By tracking evolution with copper and zinc, it may be possible to identify each of these small effect mutations, along side those final stage large effect changes present at the final time point.

We have here been interested in determining whether NGH has evolved in response to evolution in a stressful environment, only briefly moving to confirm the predicted fitness benefit that this brings. It has previously been found that higher heterogeneity strains can outcompete lower heterogeneity strains when put in direct competition in *S. cerevisiae* (Acar, *et al.,* 2008). To compare the fitness of the parental strain to an evolved state, between those populations evolved with or without stress, or even between different evolutionary outcomes, a competition study such as that used by Lenski *et al.* (1991) could be used (Figure 22). In this case, red and white colonies correspond to Ara^- and Ara^+ phenotypes, respectively, when spread on tetrazolium arabinose (TA) indicator plates. As both isolates have the marker inserted at the same location, any fitness effect will be seen equally in both isolates. The individual isolates with their respective markers are cultured individually, before mixing in a 1:1 ratio. After a set time period, one isolate is likely to outcompete the other. When plated on the TA plates, the exact ratio can be quantified. Here we could use this method to provide a quantifiable relative fitness value related to the evolution of NGH.

We should consider populations as needing to balance costs under standard conditions with benefits during stress. As such, NGH can be seen as a bet-hedging technique (Beaumont, *et al.,* 2009; Levy, *et al.,* 2012). While it has been suggested that environmental perturbation and stress is linked to a reduction in genetic variation (MacDougall, *et al.,* 2013), here it is suggested that the same circumstances can in fact lead to an increase in NGH under certain conditions. Evolution and selection therefore play an essential role in the increase of bet-hedging in a population.

The findings presented here provide new information that can add to our current understanding the various survival mechanisms that underlie microbial persistence and evolution in stressful environments. Such information, along with future advances that the creation and storage of such strains will allow, will prove useful both in a medical context, and in the context of environmental change. Valuable resources have been produced in the form of the single celled *S. cerevisiae* and the multi-cellular *C. elegans* populations evolved under known conditions with either copper or zinc stress. Once SNPs have been identified in the easy to handle yeast, homologues can be targeted in higher organisms such as nematodes, for a more complete picture of the specific mechanisms of NGH. Stressful environmental conditions driven by pollution and climate change are common in the world in which we currently live, and we can only understand the true impact of these changing environments if we examine the genetic and phenotypic impacts on the smallest of organisms, and linking these findings all the way through to the largest.

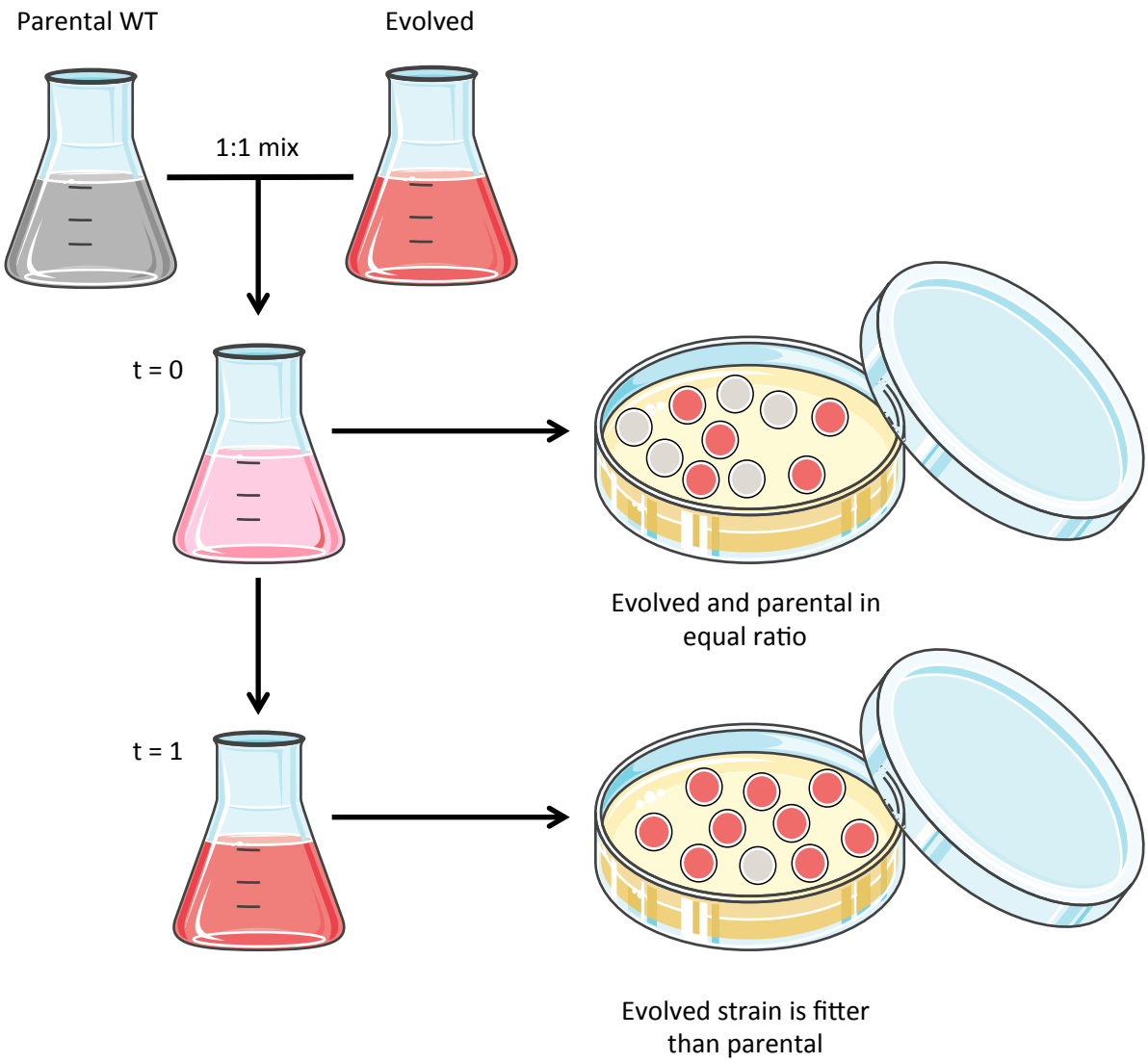

Figure 19. Schematic of a competition study such as that used by Lenski *et al.* (1991). In this case, red and white colonies correspond to Ara^- and Ara^+ phenotypes, respectively, when spread on tetrazolium arabinose (TA) indicator plates. When plated on the TA plates, the exact ratio can be quantified, giving a relative fitness value.

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APPENDIX I

Isolates evolved without copper

	Kill Curves							
	Hillslope	**|Hillslope|**	**log|Hillslope|**	**IC50**	**Mean**	**SD**	**CV**	**CFU**
X1-1	-349.3	349.3	2.543	9.384	1.923	0.473	0.246	79
X1-2	-199.0	199.0	2.299	9.117	2.556	0.941	0.368	64
X1-3	-312.3	312.3	2.495	9.134	3.110	0.672	0.216	62
X1-4	na	na	na	na	2.623	0.607	0.232	94
X1-5	-126.2	126.2	2.101	9.119	3.074	0.818	0.266	82
X1-6	-22.1	22.1	1.343	11.020	3.527	0.675	0.191	56
X1-7	-17.7	17.7	1.249	11.880	3.624	0.843	0.233	51
X1-8	-33.1	33.1	1.520	13.020	3.490	0.868	0.249	72
X1-9	-24.5	24.5	1.390	11.680	3.810	1.125	0.295	65
X1-10	-10.6	10.6	1.024	9.768	4.257	1.157	0.272	62
X2-1	-46.8	46.8	1.670	9.924	3.046	0.758	0.249	68
X2-2	-27.5	27.5	1.439	9.716	2.507	0.544	0.217	59
X2-3	-36.3	36.3	1.560	9.734	3.621	0.820	0.227	56
X2-4	-13.4	13.4	1.128	8.835	4.546	0.945	0.208	60
X2-5	-10.4	10.4	1.017	8.180	4.613	0.994	0.216	58
X2-6	na	na	na	na	3.558	0.760	0.214	50
X2-7	-313.5	313.5	2.496	9.401	3.950	0.981	0.248	63
X2-8	na	na	na	na	4.267	1.061	0.249	70
X2-9	na	na	na	na	4.362	0.840	0.193	68
X2-10	na	na	na	na	4.188	1.219	0.291	59
X2-11	-121.3	121.3	2.084	7.231	na	na	na	na
X2-12	-356.2	356.2	2.552	7.047	na	na	na	na
X2-13	-278.0	278.0	2.444	7.012	na	na	na	na
X2-14	-351.4	351.4	2.546	7.042	na	na	na	na
X2-15	-351.4	351.4	2.546	7.042	na	na	na	na
X6-1	-18.7	18.7	1.272	11.510	3.751	0.556	0.148	53
X6-2	-13.6	13.6	1.134	10.720	2.801	0.698	0.249	74
X6-3	-14.2	14.2	1.153	8.842	3.173	0.766	0.241	78
X6-4	-16.6	16.6	1.220	10.000	3.967	0.870	0.219	62
X6-5	na	na	na	na	5.630	2.414	0.429	62
X6-6	-38.2	38.2	1.582	9.279	4.040	0.832	0.206	43
X6-7	-21.2	21.2	1.325	9.498	3.339	0.834	0.250	80
X6-8	-23.9	23.9	1.378	9.438	3.685	0.937	0.254	77
X6-9	-9.2	9.2	0.965	8.468	4.429	1.161	0.262	58
X6-10	-26.6	26.6	1.425	11.290	4.570	1.467	0.321	56
X6-11	na	na	na	na	na	na	na	na

Isolates evolved with copper

	Kill Curves							
	Hillslope	**|Hillslope|**	**log|Hillslope|**	**IC50**	**Mean**	**SD**	**CV**	**CFU**
Cu3-1	-17.5	17.5	1.244	10.760	1.400	0.655	0.468	65
Cu3-2	-20.9	20.9	1.319	12.500	1.424	0.416	0.292	79
Cu3-4	-15.9	15.9	1.201	12.300	1.740	0.428	0.246	91
Cu3-5	-21.9	21.9	1.341	9.658	1.609	0.530	0.329	111
Cu3-6	-14.1	14.1	1.149	9.810	1.911	0.679	0.355	79
Cu3-7	-16.6	16.6	1.221	9.165	2.553	1.061	0.415	68
Cu3-8	-14.1	14.1	1.148	11.450	3.061	0.698	0.228	79
Cu3-9	-13.1	13.1	1.118	11.820	2.618	0.605	0.231	77
Cu3-10	-8.7	8.7	0.939	9.505	3.169	0.960	0.303	71
Cu3-11	-8.6	8.6	0.937	10.490	1.583	0.479	0.302	97
Cu3-12	na	na	na	na	na	na	na	na
Cu6-1	-37.4	37.4	1.572	13.690	1.455	0.847	0.582	72
Cu6-2	-30.0	30.0	1.477	12.440	1.949	0.528	0.271	88
Cu6-3	-23.1	23.1	1.363	12.860	2.271	0.583	0.257	68
Cu6-4	-17.6	17.6	1.246	11.710	2.306	0.579	0.251	87
Cu6-5	-17.6	17.6	1.246	11.710	2.168	0.503	0.232	96
Cu6-6	-20.2	20.2	1.305	10.210	2.685	0.636	0.237	73
Cu6-7	-27.1	27.1	1.433	12.920	1.139	0.261	0.229	104
Cu6-8	-11.6	11.6	1.063	12.060	1.435	0.601	0.419	85
Cu6-9	-9.2	9.2	0.963	10.610	2.849	1.398	0.491	62
Cu6-10	-7.8	7.8	0.890	8.634	3.503	1.826	0.521	75
Cu10-1	-12.1	12.1	1.084	14.260	1.439	1.092	0.759	75
Cu10-2	-13.2	13.2	1.122	14.550	1.127	0.413	0.367	45
Cu10-3	-21.4	21.4	1.330	13.440	1.434	0.826	0.576	59
Cu10-4	-20.1	20.1	1.304	13.660	1.271	0.433	0.341	108
Cu10-5	-10.6	10.6	1.025	12.260	2.823	0.665	0.236	82
Cu10-6	-13.8	13.8	1.140	11.580	1.473	0.326	0.221	70
Cu10-7	-15.6	15.6	1.193	13.040	3.085	0.994	0.322	58
Cu10-8	-15.3	15.3	1.185	12.940	3.767	1.264	0.335	58
Cu10-9	-13.2	13.2	1.121	11.170	5.278	2.095	0.397	71
Cu10-10	-6.1	6.1	0.788	10.760	4.013	1.039	0.259	56

WT Parental Isolates

Kill Curves

	Hillslope	|Hillslope|	log|Hillslope|	IC50	Mean	SD	CV	CFU
WT-1	-32.0	32.0	1.506	8.966	1.636	0.408	0.250	65
WT-2	-36.5	36.5	1.563	9.291	1.600	0.434	0.274	72
WT-3	-220.8	220.8	2.344	8.135	1.459	0.409	0.280	69
WT-4	na	na	na	na	na	na	na	na
WT-5	-20.5	20.5	1.311	9.773	na	na	na	na
WT-6	na	na	na	na	na	na	na	na
WT-7	-456.4	456.4	2.659	10.680	na	na	na	na
WT-8	-113.6	113.6	2.055	10.310	na	na	na	na
WT-9	-579.6	579.6	2.763	10.770	na	na	na	na