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Abstract

Introduction: Decompression sickness (DCS) is a complex and poorly understood systemic disease caused by inadequate desaturation following a decrease of ambient pressure. Strong variability between individuals is observed for DCS occurrence. This raises questions concerning factors that may be involved in the inter-individual variability of DCS occurrence. This study aimed to experimentally assess the existence of heritable factors involved in DCS occurrence by selectively breeding individuals resistant to DCS from a population stock of wistar rats.

Methods: 52 males and 52 females Wistar rats were submitted to a simulated air dive known to reliably induce about 63% DCS: compression was performed at 100 $kPa.min^{-1}$ up to 1000 kPa absolute pressure before a 45 min long stay. Decompression was performed at 100 $kPa.min^{-1}$ with three decompression stops: 5 min at 200 kPa, 5 min at 160 kPa and 10 min at 130 kPa. Animals were observed for one hour to detect DCS symptoms. Individuals without DCS were selected and bred to create a new generation, subsequently subjected to the same hyperbaric protocol. This procedure was repeated up to the third generation of rats.

Results: As reported previously, this diving profile induced 67% of DCS, and 33% asymptomatic animals in the founding population. DCS/asymptomatic ratio was not initially different between sexes, although males were heavier than females. In three generations, the outcome of the dive significantly changed from 33% to 67% asymptomatic rats, for both sexes. Interestingly, survival in females increased sooner than in males.

Conclusion: This study offers evidence suggesting the inheritance of DCS resistance. Future research will focus on genetic and physiological comparisons between the initial strain and the new resistant population.

Keywords: Selective breeding, decompression illness, diving, inheritance

Introduction

Decompression sickness (DCS) is a hazard for divers. Its systemic pathology is characterized by a wide variety of symptoms, occurring among people exposed to pressure differentials (scuba divers, airplane pilots, hyperbaric chamber workers or astronauts). Among recreational divers, DCS consists of muscular and articular pain, skin rash and/or neurological impairments such as paresthesia and distal numbness. However, DCS may sometimes have more severe consequences and trigger even coma and death (30). It is well recognized that large inter-individual variability exists for susceptibility to DCS (16, 17). The risk of DCS correlates with the amount of circulating venous gas emboli (VGE) formed during and after decompression (10). However, high VGE levels have been reported post-dive without inducing DCS symptoms (2), and it has been estimated that only 13% of DCS occurrence can be explained by venous bubbles (18). This strongly suggests that other factors modulate VGE potency to trigger DCS.

Possible mechanisms include platelet activation and coagulation cascade (3, 14), inflammation (22, 26, 29), release of microparticles (27, 37), the NO-cGMP pathway (19, 35), and the renin-angiotensin aldosterone system (20). However, even if these factors appear to be linked to DCS occurrence, none of them seem to be determinant in the onset of this disease and the precise pathophysiological sequence of events leading to DCS is still to be described. The use of a non *a priori* approach, such as recently used (15), may help identifying new physiological or genetic factors.

Many risk factors have been linked with DCS susceptibility. Various non alterable risk factors have been described, such as a patent foramen ovale (PFO) (12, 28), age (4, 7) and sex (6). There are also alterable factors such as body mass (24) and $\dot{V}o_2max$ (34, 36). Even if these factors may be improved by a proper training, they may have a heritable component that could, at least in part, explain DCS susceptibility, which has not previously been investigated before. The present study aimed to experimentally assess whether susceptibility to DCS includes a component of inheritability by selectively breeding resistance to DCS from a population stock of wistar rats.

Methods

The protocol described in this study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996) and was approved by the Université de Bretagne Occidentale Ethics Committee for Animal Experimentation (approval no.5250).

Founder population

The two most common strains of rat used in decompression research are the outbred strains Wistar and Sprague-Dawley (6). There is no difference in their susceptibility to DCS following the compression-decompression protocol used in this study (6). 52 male and 52 female Wistar rats (n=104) aged 6 weeks old were obtained from Janvier SAS (Geneste, France). The Wistar strain was chosen because, although its overall heterozygosity is less than in wild rats, it is still more than is found in its Sprague-Dawley counterpart (13). Animals were housed until the day of the hyperbaric protocol at the university animalry three per cage under controlled temperature (21 ± 1°C) and lighting (12 h of light per day, 0600–1800). They were fed standard rat chow and

water *ad libitum*. In order to enable individual follow up, all animals were identified with an electronic tag.

Hyperbaric protocol

At 11 weeks old (80±1 day old), rats from the initial lot were transferred to the hyperbaric chamber and rested for 1 hour before being subjected to a standard hyperbaric protocol dive known to trigger 63±4% DCS(6). Three cages of rats at a time were placed in a 130L steel hyperbaric chamber and then compressed with air to 1000kPa absolute pressure (equivalement to 90msw) at a rate of 100kPa.mn^{-1} and maintained at maximum pressure for 45mn (Figure 1). The chamber was then decompressed at a rate of 100kPa.mn^{-1} with 3 stops (5mn at 200kPa, 5mn at 160kPa and 10mn at 130kPa). Total hyperbaric exposure duration was 83mn. All simulated dives were performed in the morning (at 8, 10 and 12AM), to avoid circadian differences due to day/night cycle, and within one week to avoid any difference in age (animals were 80±1days old at the time of the dive). Three simulated dives were performed each day, during which 9 rats were exposed each time. This allowed testing a maximum of 135 animals for DCS resistance per week. Compressions were spaced 2 hours apart (30mn at rest, 83mn hyberbaric exposure, 5mn chamber cleaning).

Selection protocol

After decompression, animals were observed for one hour for signs of DCS, especially respiratory distress, paralysis, convulsions and death. This observation method has already been used in previous studies (1, 17). During this observation period, any rat with DCS was anaesthetized and euthanised by interperitoneal injection of ketamine/xylazin, whereas

asymptomatic individuals were taken back to the university animalry to recover for a week before mating. Thus only rats which displayed no visible signs of DCS were considered asymptomatic.

Among asymptomatic individuals, 13 couples were then selected for mating. Pups born to these couples comprised the next generation. Newborns were housed in the same conditions as their parents, identified with an implanted electronic tag at the age of 4 weeks, subjected to the same hyperbaric protocol when 11 weeks old and, then, to the same selection protocol. Couples were numbered from C01 to C13 to allow tracking ancestry and avoid inbreeding. At each pairing each male was paired with females from consecutive families. So, the resistant male from family 1 (maleC01) was paired with the resistant female from family 2 (femaleC02), then for the next generation the resistant male from family 1 (maleC01) was bred with the resistant female from family 3 (femaleC03) (Figure 2).

Statistics

The initial sample size was chosen to ensure at least 13 surviving rats from each sex with an error margin of 20%. This sample size also ensured a power to detect of 0.8 (α=0.05): with a diving profile leading to 60% DCS occurrence, a drop to 40% or less DCS was needed to show a statistically significant gain in resistance to DCS between the initial lot and their descendants. The proportion of DCS outcomes between generations was evaluated by Chi^2 tests. Potentially relevant factors including day and time of exposure, age and body weight were evaluated using logistic regression and potential interactions between age, weight and generation were also assessed for significant association with DCS outcomes. The models were optimized using the

likelihood ratio test. Differences in mean weight across three generations were assessed using ANOVA. Upon significant differences in the ANOVA, Tukey post-hoc test was used to assess differences. Binary groups were compared using T-tests. All data were stored in MS Excel and analysed using the statistical program R.

Results

DCS in the 1st generation

The first generation (G1) showed a DCS prevalance of 65% for both males and females (Figure 3), although males were significantly heavier than females (426±24g vs 251±17g respectively, independent t-test, $p=2.2.10^{-16}$). As shown in Figure 4, in this first generation body weight was not significantly different between males that suffered DCS or were asymptomatic ($p=0.63$) and the same was true for females ($p=0.22$), suggesting that body weight did not impact the outcome of the dive within either sex in the founding animals.

Effect of the selection protocol

As shown in Figure 3, the second generation (G2) showed a significant decrease in DCS prevalence among females (33% against 65% for G1). DCS prevalence was not significantly different between G2 males and G1 males. However, males from the third generation (G3) showed a significantly decreased DCS prevalence (35% against 68% for G2). Interestingly, females from G3 showed no significant decrease of DCS prevalence compared to G2.

Age in days was included in the logistic regression to assess whether age (rats were at 80±1days old at the time of the dive) elicited changes in DCS occurrence. Body weight was also included, as well as sex. Day and hour of exposure to pressure were also included in the initial model, as was generation. Collinearity between these factors were included in the regression as interactions age*weight, sex*weight, and weight*generation. After backwards elimination, dismissed factors included day of diving, hour of diving, age, weight*Generation and age*weight. Hence, our final logistic model described DCS probability as shown in Equation 1.

$$Logit\ (DCS) \sim -7.57 + 6.18(Male) + 0.0315(Weight) - 0.509(G2) - 1.24(G3) - 0.0259(Weight|Male) \quad (1)$$

P-values and odd ratios for significant variables are presented in Table 1. G3 was highly significant, indicating an inheritability of DCS resistance. Moreover, sex was also statistically significant and indicated that the evolution of DCS resistance differed between males and females. Taking into account this strong involvment of sex on DCS outcome, logistic regressions were then conducted separately for each sex following a similar model optimisation method.

Concerning males, the final logistic model is shown in Equation 2.

$$Logit\ (DCS|M) = -2.34 - 0.99(G3) \quad (2)$$

In this sex, G3 but not G2 was statistically significant (Table 1), indicating that resistance to DCS appeared in the third generation. Although body weight was significantly different between the three generations of males (ANOVA, $p= 3.5.10^{-6}$) (Figure 4), the evolution of resistance to DCS in males did not rely on weight (Table 2).

Concerning females, the final logistic model is shown in Equation 3.

$$Logit\ (DCS|F) = -7.35 + 0.0320(Weight) - 0.127(G2) - 1.41(G3) \quad (3)$$

In this sex, we found that both G2 and G3 variables reached statistical significance which indicated that resistance to DCS increased between the second and third generation (Table 2). Body weight was not significantly different between the three generations of females (Figure 4) (ANOVA, p=0.16) but the evolution in resistance to DCS was nonetheless linked to body weight in all generations (Table 2).

Narrow sense heritability using a parent-offspring regression (11) was computed for G2 and G3. The transition from 35% resistant to 50% resistant gave a h2 of 0.23 for G2 rats. The transition from 50% to 69% gave a h2 of 0.39 in G3 rats.

Discussion

This study is the first to report data showing inheritance of resistance to DCS. Indeed, DCS resistance doubled in a matter of only two generations. Females showed a steep rise in DCS resistance between G1 and G2, whereas males showed DCS resistance rise between G2 and G3.

DCS in the 1st generation

As mentioned in the results, the first generation showed the expected DCS ratio of 65% for males, which agrees with previous data from our group (7). Interestingly, females showed the same DCS outcome, even though they were 175g lighter than males. According to previous studies which clearly demonstrated that body weight is a risk factor for DCS in rats (17), females should have suffered less DCS than males. Indeed, Buzzacott et al.(6) estimated an odd ratio of 1.02 for weight in a meta-analysis which included both males and females. Given this, the 175g difference between males and females should have led to a higher incidence of DCS in males than in females which did not happen. This result suggests that being a female was a risk factor itself in the stock population, as previously described(19, 23). This result is surprising because it has been previously shown that women may be less prone to VGE formation than men under certain circumstances (5). This reinforces the hypothesis that VGE is only a part of the mechanism of DCS. On the other hand, weight did not significantly influence DCS outcome in our study when considering each sex separately. This apparent discrepancy likely results from the very small range for mean weight per generation (no more than 47g within males and 34g within females, 95% Confidence Interval).

Effect of the selection on DCS resistance

Variables included in the logistic regression were selected according to their anticipated impact on DCS occurrence. Additional variables were added to the model to account for the presence of collinearity. Age(7) is known to influence the outcome of DCS. Circadian rhythm exerts an influence on many physiological functions(21, 33) which may possibly influence the development of DCS. For these reasons, we included the exact age as well as the timing (in the

day and in the week) each rat was exposed to the hyperbaric protocol. Logistic regression showed that all these methodological factors such as the small difference in age between animals on the day of exposure to pressure (80±1days old), day in the week and hour of diving had litte impact on the incidence of DCS. Therefore, although animals were not exposed to the hyperbaric protocol at the same moment of the day and of the week, we did not detect any bias due to methodological causes which could have artificially influenced our results.

Interestingly, generation was significant in both males and females. Although weight was significantly different in male rats across generations, it was not a significant factor in the logistic regression ($p=0.11$), suggesting that the decrease in DCS occurence in the third generation was not due to a decrease in weight. This is confirmed by the lack of correlation between DCS and weight. Conversely, weight was still significant in female rats ($p=0.0007$). Females were similarly homogeneous in weight accross generations as males (404±40g for males and 246±19g for females respectively). Thus, weight involvement in the female group cannot be explained by greater weight heterogeneity in female rats than in males and suggests that the mechanisms implied in the development of DCS are different in females and males. Furthermore, weight was significantly different between asymptomatic and females which suffered DCS, but not between generation. With an OR of 1.03, weight for females could explain about 40% of DCS occurrence, versus 76% explained by generation (OR=1.24). Furthermore, the interaction variable between generation and weight was eliminated during model optimisation. Taken together, these observations rule out the possibility that the decrease in susceptibility to DCS resulted from selection of the lightest individuals and strengthens the evidence of an independent effect of breeding on DCS resistance. We observed a quick and

asymmetric raise of asymptomatic males and females (35 to 67% between G1 and G2 females, and 32 to 65% between G2 and G3 males). Transmission of a specific X-linked trait, giving a strong advantage in the face of extreme environmental stress, could be expected to demonstrate progressive adaptation in a manner comparable to that observed in this study (8). While this hypothesis remains speculative till further investigation, it is conceivable from our data to describe DCS resistance as potentially linked to a gene or a major gene group, borne by chr.X in the rat, as highlighted by the steep rise of DCS resistance in females firstly, followed by males. Many genes located on the X chromosome code for proteins known to be involved in the development of DCS, such as Angiotensin converting enzyme(20), NFKB pathway activating or repressing proteins or CD40 ligand(9). However, other heritable transmission paths are possible, such as by mitochondrial DNA if DCS resistance is linked to the electron transport chain(25, 32) or by epigenomic regulations borne by the mother(38). Further research is needed to investigate these hypotheses, to identify which genetic mechanisms confer resistance to DCS.

Limitations

Although the results presented in this study appear to point out a possible genetic determinism of DCS occurrence, some limitations must be addressed. As stated previously, the Wistar strain, albeit outbred, still has limited overall genetic variability compared with wild stock. This limits the possibility of detecting genuine effects of DNA variation, especially in a small number of generations, and could explain these surprising results, given that DCS is commonly admitted as a complex trait. However, this study nonetheless points out a previously unknown heritable componant in the variability of DCS susceptibility displayed by Wistar rats, which deserves to be further investigated. The lack of a control line is one of the limitations of

this study. Unfortunately, in our opinion a line of control rats would have been problematic to obtain due to methodological issues. Namely, to breed a control line exposed to the breeding conditions only without any selection process would dismiss the effects of diving itself which was found to have significant long term impact (9). Alternatively, a control "diving" strain would require a benign diving protocol such that no rat would have suffered DCS by using a different diving profile. A shallower and/or shorter compression/decompression profile with less decompression stress would consequently expose the rats to lower level and/or duration of hyperoxia, whereas a profile with longer decompression stops (and, therefore, also less decompression stress) would include a longer period of hyperoxia. In short, the interactions of hyperbaria and hyperoxia cannot be replicated in a benign dive profile without exposing the rats to equal decompression stress, which would preclude breeding a "true" control line. Indeed, it has been showed that hyperoxia interacts with hydrostatic pressure to stimulate the production of Reactive Oxygen Species and consequently could have a significant impact (31). Instead, we opted to not choose between these two "imperfect control lines" and to compare the resultant line of resistant rats to both the founding stock, which are preserved for this purpose, and with similar outbred Wistar rats from the same supplier as the founding population were obtained from. Even without a true control line, the steep rise of DCS resistance between generations points to an independent effect of the selection protocol. Further research will investigate the mechanisms of this inheritable trait.

Conclusions

In conclusion, the present study showed that it is possible to raise DCS resistance among a rat population within two or three selectively bred generations only and that, in samples with homogenous weight, DCS resistance was first expressed in female rats. This finding shows the involvement of innate factors that may be transmitted by DNA, mother mDNA or mother epigenomic regulations. The nature of these inherrant trait remains to be investigated in further studies.

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Conflict of interest

The authors declared no conflict of interest. The results of the present study do not constitute endorsement by ACSM. The results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

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Captions

Figure 1: Compression/Decompression profile of the simulated air dive. The profile consists of a compression phase of 100kPa.mn^{-1} up to 1000kPa. Bottom phase lasts 45m, followed up by the ascent phase of 100kPa.mn^{-1}. The profile ends with 3 decompression stops: 5mn at 200kPa, 5mn at 160kPa and 10mn at 130kPa.

Figure 2: The process used to selectively breed decompression sickness resistant rats.

Figure 3: Asymptomatic animal frequency by generation and sex. Proportion of asymptomatic animals was significantly different between G1 and G2 females (Chi^2 $p=5.4.10^{-4}$), and between G2 and G3 males (Chi^2 $p=2.6.10^{-4}$).

Figure 4: Mean weight in grams by generation and DCS outcome in females (A) and males (B). For females, weight was not different between generations (ANOVA p=0.16), but was different between conditions ($p=4.2.10^{-3}$). For males, weight was different between generations (ANOVA $p=3.5.10^{-6}$), with a difference between G1 and G2 (post-hoc,p=0.00271), G1 and G3($p=1.10^{-4}$) G2 and G3 (p=0.03). However, weight was not different between conditions when taking into account the generation (ANOVA p=0.27).

Figure 1

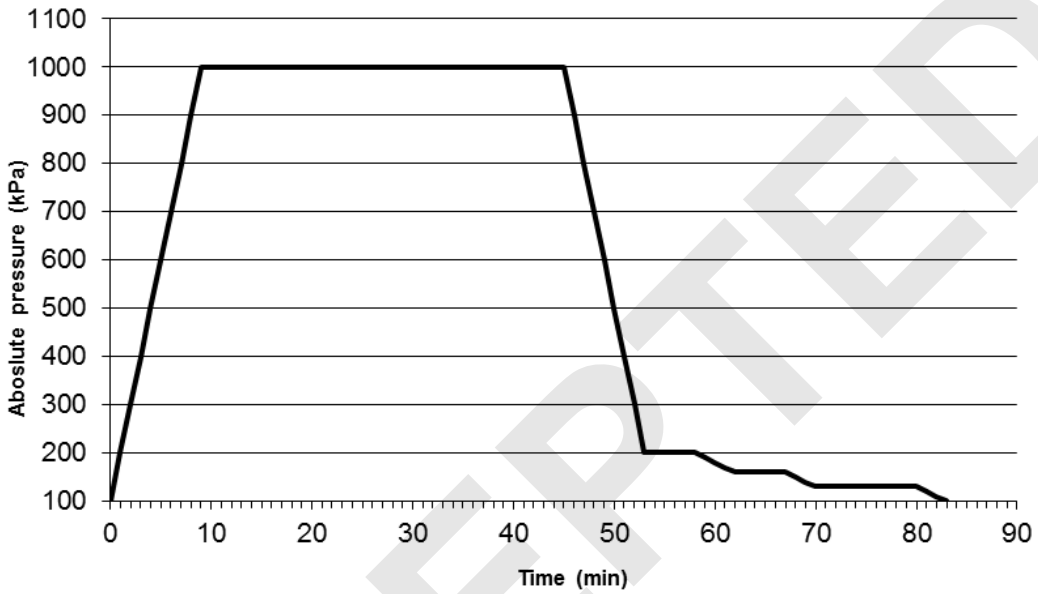

Figure 2

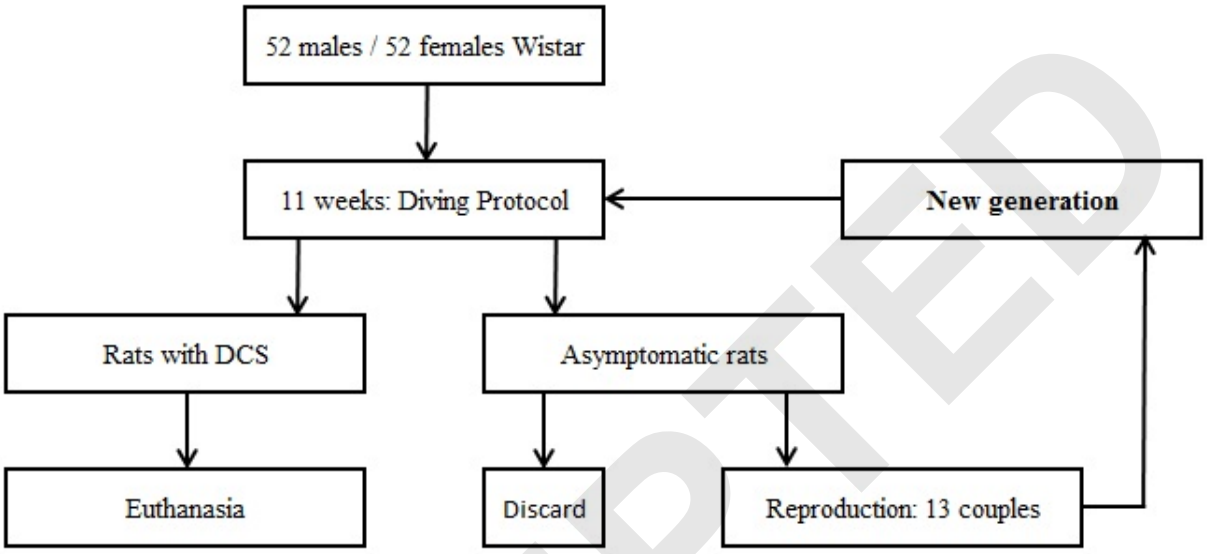

Figure 3

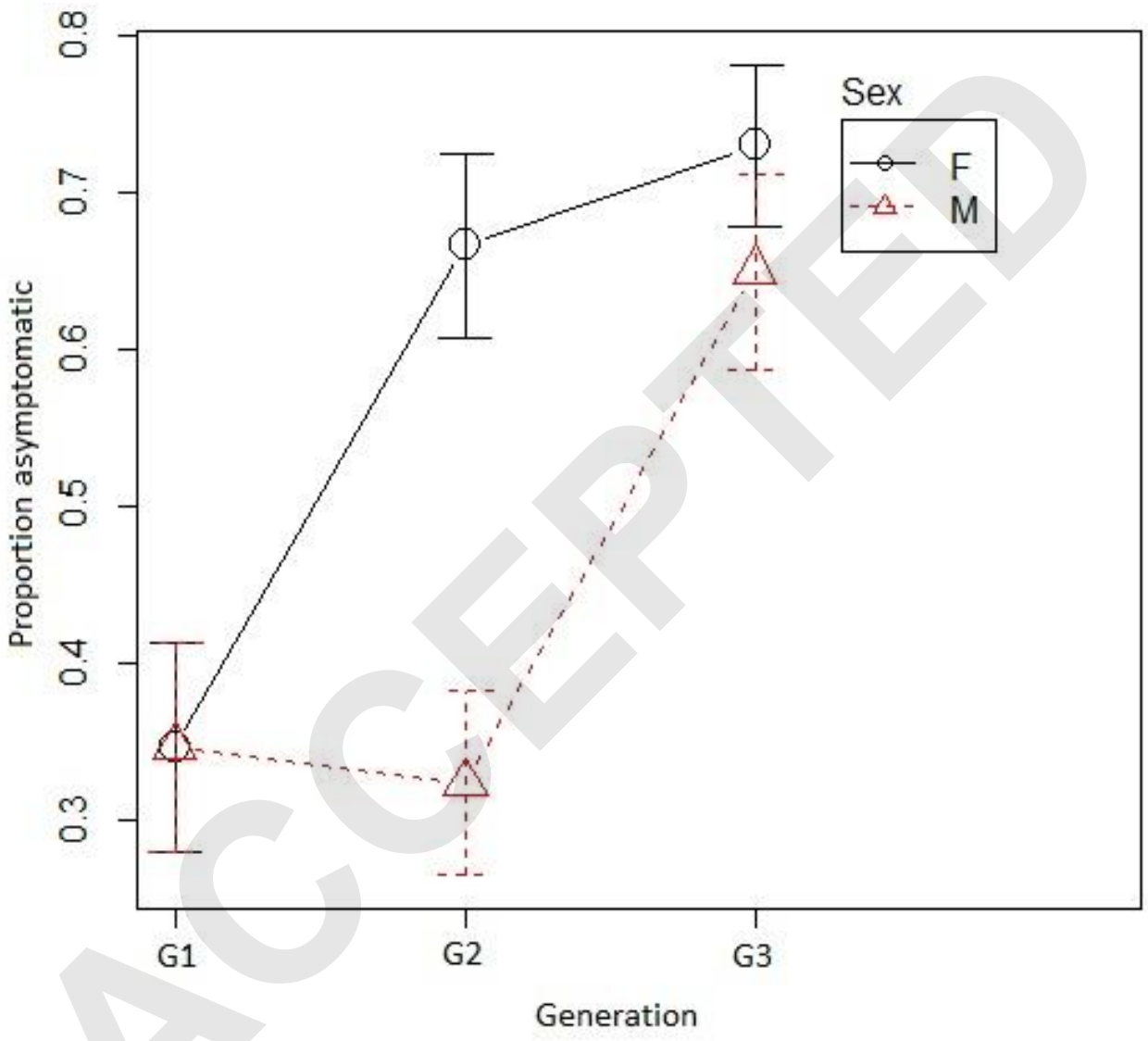

Figure 4

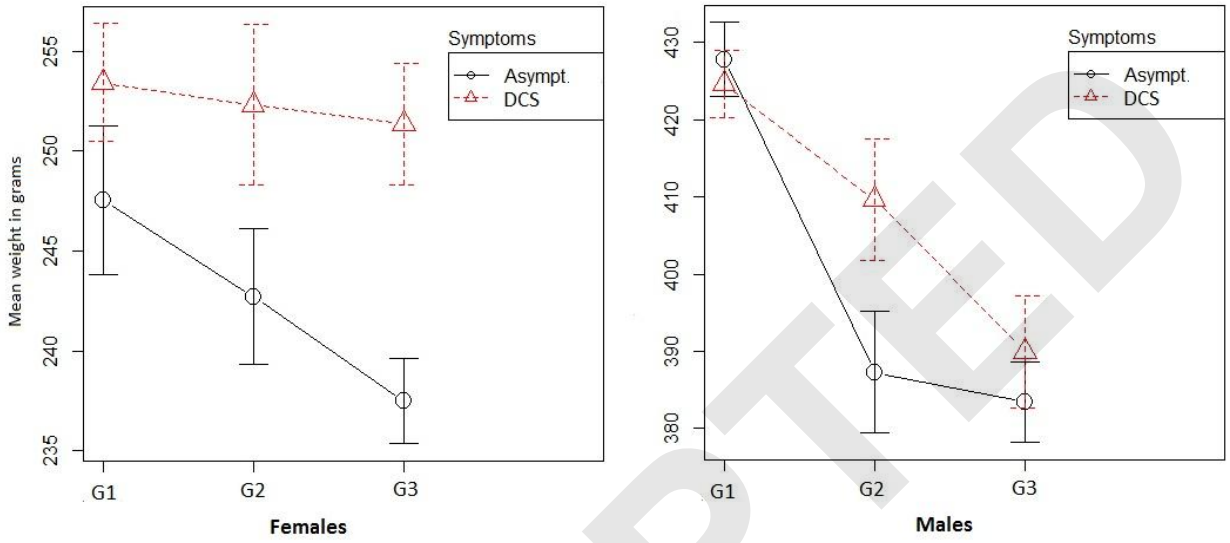

Table 1: Odds ratio for each factor involved in DCS occurrence. Significant p values are in bold. G2 and G3 respectively stand for Generation 2 and Generation 3.

Factors	***Odds Ratio (OR)***	***P value***
Sex	483	**0.028**
Sex*Weight	0.97	**0.01**
Weight (g)	1.03	**0.0007**
G2	0.60	0.073
G3	0.29	**$3.2.10^{-5}$**

Table 2: Odds ratio and significance of each factor in the optimised models. Significant p values are in bold.

Factors	***Odds Ratio (OR)***	***P value***
Weight (g)		
Males	N/A	0.11
Females	1.03	**0.0007**
G2		
Males	N/A	0.50
Females	0.28	**0.002**
G3		
Males	0.37	**0.02**
Females	0.24	**$4.8.10^{-4}$**