Maximizing the max test: Development of a maximal graded exercise test for the assessment of cardiovascular function in mice

THESIS

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Abstract

Tools for the functional assessment of cardiovascular fitness are needed to establish animal models of dysfunction and the effects of novel therapeutics. Currently, exercise assays are widely published; however, they have limited sensitivity for assessing the cardio-metabolic phenotype of mice. In human research, the graded maximal exercise test (GXT) is a gold standard diagnostic for measuring cardiovascular function. Present published testing methods in mice use set inclination and progressive increases in speed until exhaustion (PXT); thus lacking characteristics of the GXT. We developed a GXT test that allows for assessment of cardiovascular fitness in metabolic and genetic models of cardiovascular dysfunction; as well as in healthy mice. The results of comparison between this method and the PXT revealed that only the GXT test provides sensitive, quantitative, parameters for diagnosing and monitoring cardiovascular, metabolic and pulmonary function in mouse models.

Dedicated to my parents, Linda and Vincent Petrosino. Without their unconditional love and support, none of my accomplishments would have been possible.

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Publications

1. **Petrosino, J.M**.; DiSilvestro, D.; Ziouzenkova, O. Aldehyde Dehydrogenase 1A1: Friend or Foe to Female Metabolism? (2014). *Nutrients*, *6*, 950-973

2. DiSilvestro, D.; **Petrosino, J.M**,; Aldoori, A.; Melgar-Bermudez, E.; Wells, A.; Ziouzenkova, O. (2014). Enzymatic intracine regulation of white adipose tissue. *Horm Mol Bio Clin Invest*, *19(1)*, 39-55

Fields of Study

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Chapter 1: Introduction

At a time where the obesity epidemic rises and heart disease remains the leading cause of death, a large interest has arisen to better understand the intersection between metabolic and physiological mechanisms regulating cardiovascular function and energy metabolism (reviewed in[2-5]). One of the most powerful tools to better understand this intersection has been the mouse model. Through the overexpression, knockdown, or knockout (KO) of specific genes, we now have the ability to study the effects of specific cardiovascular and metabolic genes *in vivo*[6-8].

With the increased prevalence models also comes the increased need to functionally assess the cardiovascular and metabolic phenotypes of these mice[9, 10]. Thus, it is no surprise that the popularity of using mouse models in basic science research has been accompanied by an exponential increase in the use of exercise assays as functional assessments of mouse cardiorespiratory fitness[11-15].

The mouse, which shares many metabolic, anatomic, and genetic similarities with man[16], is a model organism to study metabolism and physiology *in vivo*.

Exercise testing has served as a prototypical method for the study of functional interactions between the cardiovascular, pulmonary and muscular systems under stress (deemed cardiorespiratory fitness) in man for over 50 years[1, 17, 18]. Accordingly, basic science cardiovascular research in the 1980s, 1990s, and 2000s followed suit[12, 19-21] and numerous studies were done reporting basal and exercise metabolic and cardiovascular testing in mice[11-15,11, 12, 21, 22]. However, with this increased use of exercise testing also came controversy related to data interpretation, positive test criteria, and failure to reference classical literature from the exercise physiology field (reviewed in[23, 24]).

In this thesis we describe our comparative approach to the generation of an improved initial and continual assessment of cardiorespiratory fitness in functional and dysfunctional mouse models. Experiments in our lab utilizing exercise assays based on previous research led us to believe that there was a need for a more sensitive type of assay to determine cardiovascular fitness in mice using exercise testing. Our hypothesis was that we could improve the ability of the exercise assay to determine the cardiovascular fitness of a mouse by integrating criteria of human graded maximal exercise testing (GXT) and specific considerations to mouse physiology. Thus, while this research arose from the need for a more sensitive assay to test the cardiovascular function of a

previously uncharacterized mouse model; it later developed into the generation of an improved exercise test capable of characterizing the cardiovascular, pulmonary, and metabolic phenotype of functional and dysfunctional mice.

Chapter 2: Review of Literature

2.1 Introduction

In 1946 the first exercise protocol was used in mice[25]. Since then, over 5,000 publications have included the use of a mouse exercise assay. Cardiovascular disease (CVD)[26] and obesity[27] are two of the most prevalent diseases in the United States and intersect at their relationship between fat accumulation, cardiac function, and inactivity[28]. As a result, use of exercise testing as a way to functionally assess mouse cardiovascular and metabolic phenotypes has skyrocketed. From 2000-2014, over 125 papers were published in journals with impact factors over 30 utilizing mouse exercise protocols. Furthermore, from January to April of 2015, over 28 papers using animals[29-56] have implemented the use of a treadmill-based exercise assay.

2.2 Types of assays previously used in the literature

A wide variety of testing exists in the literature to measure the cardiovascular fitness of a mouse. While some have used methods like swimming[57] and voluntary wheel running[13, 15, 58-60], the majority used treadmill testing. Many

previously performed assays utilize a fixed inclination but progressively increase in speed until exhaustion is achieved and VO_{2max} is recorded[13-15, 20, 58-67]. With these protocols, set inclination differs anywhere between approximately 0˚ and 30˚. Others have show[63] in trained and untrained mice that higher fixed inclinations can result in improved maximal oxygen consumption (VO_{2max}) and peak respiratory exchange ratio (RER) values. This notion, that inclination affects oxygen (O_2) uptake during maximal aerobic exercise, is also supported in human research.[68] Human VO_{2max} testing has been a fundamental measurement to assess aerobic and cardiovascular function in human subjects sine the 1900's [69]. Many of these protocols, like the Bruce[70], differ from these animal exercise assays in that they increase in speed and inclination over the course of various stages of testing.

2.3 Purpose of exercise testing

Cardiorespiratory fitness, the combined functional state of cardiovascular, pulmonary and skeletal muscle systems[18], is functionally measured in clinical and laboratory settings through the use of maximal and submaximal VO_{2max} testing[1, 18]. These tests are preformed in general[71], athletic[72], and diseased human populations[70] and can include measures of oxygen uptake (VO_2), carbon dioxide output (VCO_2), blood pressure, heart rate, minute ventilation (VE), arterial blood

gases, and other variables derived from a 12-lead echocardiogram (EKG)[1, 18]. While both treadmills and cycle ergometers can be used to perform these tests[73-79], a graded VO_{2max} treadmill test (GXT) known as the Bruce Protocol[70] is considered the gold standard in the United States[17, 77] for the assess cardiorespiratory fitness and initially diagnosing coronary artery disease (CAD)[80]. More specifically though, these tests can be used to identify: myocardial ischemia, prediction of cardiovascular events, evaluation of aerobic exercise capacity and tolerance, responses to therapeutics, and assessment of chronotropic competence[81]. Thus, to best retrieve diagnostic and prognostic information from these tests, one needs to understand the purposes of utilizing them and accordingly consider appropriate end points and modalities.

2.4 The value of using gas exchange analysis retrieved from testing

Significant amounts of information can be obtained when a VO_{2max} test includes metabolic information (deemed cardiopulmonary exercise test (CPX)) in addition to work outputs and measures from the EKG[82-84]. Functions of the cardiovascular system are not limited to cardiac output; as gas exchange involves the integration of the cardiovascular, pulmonary, and musculoskeletal systems[85]. This integration forms a single circuit responsible for the exchange of gas between the external and internal environment. This gas exchange,

specifically the ability to remove CO_2 and metabolites and supply the muscle with O_2 and other fuels, is extremely important to cardiac function[86]. When a CPX is performed, VO_2 and VCO_2 are measured at the mouth and considered equivalent to cellular-level oxygen utilization (Q_{O2}) and carbon dioxide production (Q_{CO2}). At rest external respiration is equal to internal respiration; however during metabolic stress like exercise, this begins to change as the energy demands of working muscles increase[86]. VO_{2max}, the product of cardiac output and maximum ateriovenous oxygen difference ($_{a\text{-}v}O_2$ difference), is considered the best measure of exercise capacity or cardiovascular fitness but can only be directly measured through the collection of gas exchange[87]. Accordingly, it is through the collection of gas exchange data that we can better understand physiological responses occurring under metabolic and mechanical stress[81].

2.5 Criteria and considerations for generating a graded maximal exercise test in human populations

While treadmills and cycle ergometers are the most commonly used modalities for graded maximal exercise test (GXT), much consideration goes into selecting the appropriate protocol to test patients. Protocol selection is not haphazard and should be mandated by patient conditions and testing objectives[18, 80]. Although the Bruce is considered a gold standard, it does have some limitations in that the

large increases in workload between stages can be intolerable for obese and elderly individuals [18]. Other protocols that are still GXTs but slightly differ include the Cornell[88], with shorter stages and interpolated half stages, as well as the Naughton and Balke protocols which have reduced increases in stage to stage intensity changes[18]. Regardless of the GXT selected and the associated differential protocols used to achieve VO_{2max}, it should be noted that standardization criteria was implemented to generate these protocols.

This criteria[80] includes: (1) simultaneously progressive (incline and speed) (2) increasing heart rate with continual increases in exercise intensity (3) termination at terminal fatigue (4) a length of 8-12 minutes and (5) a significant increase in lactic acid concentrations from pre to post test. Exercise physiologists and clinicians generated this criterion with specific consideration to the physiological effects on the cardiovascular, pulmonary, and musculoskeletal systems.

GXTs consist of stages of concurrent increases in speed and incline as a way to optimally induce stress to the cardiovascular system. Stages are designed to last approximately 3 minutes so that there is stabilization of heart rate, cardiac output, blood pressure, and VE before intensity is again increased[81, 87]. Exercise fatigue, or work output that occurs prior to exhaustion, is one of the most powerful

predictors of survival from a cardiac event or likelihood of death[89, 90]. Accordingly, terminal fatigue is necessary to accurately retrieve VO_{2max}. As exercise transitions from moderate to maximally intense, cardiac output drives the associated increase in VO_{2max} and a resulting state of sympathetic dominance and parasympathetic inhibition is elicited[81]. This response induces vasoconstriction to all systems; with the exception of circulation to the heart, brain, and working muscles. Maximal exercise testing induces specific stress to working muscles and the heart. Consequently, this means that the length of the test can be both informative and limiting. Too large of increments in intensity per stage is associated with a weaker relationship with VO_2[17]. However, if the protocol is not intense enough or lasts longer than 12 minutes, exhaustion and performance are no longer the result of cardiopulmonary endpoints and instead become limited by variables such as orthopedic complications and muscle fatigue[17]. Thus, consideration that went into the development of testing criteria is anything but arbitrary.

Chapter 3: Research Manuscript

3.1 Introduction

Exercise stress testing is a key non-invasive and cost-effective method for the assessment of cardiorespiratory fitness and is an initial diagnostic of coronary artery disease (CAD)[80]. There are various graded exercise tests (GXT_h)[73-78] use to assess the combined functional state of the cardiovascular, pulmonary and skeletal muscle systems[18] (cardiorespiratory fitness); however, amongst them the Bruce Protocol[70] remains the gold standard. The development of new therapies for metabolic and cardiovascular disease has fast progressed with the development of genetic and epigenetic mouse models of cardiovascular dysfunction. These models are used to study cardiac biology, physiology, and novel therapeutics prior to translation of these findings to man[6-8, 91, 92]. As a result, methods of maximal exercise testing has been widely published[29-56] as a way to functionally characterize the metabolic and cardiorespiratory phenotype of these mice[23, 65, 93, 94]. However, unlike with human testing, there are no standardized protocols or end point criteria for positive tests. These, and other numerous limitations which question the ability of exercise assays to accurately make assessments of cardiovascular function in mice, have been and continue to

be highlighted (reviewed [23, 24]).

The majority of these assays[14, 20, 61-67] are univariate, progressive, maximal exercise tests (PXT). They include a set inclination and progress in speed until VO_{2max}, exhaustion, or both are achieved. This setting provides parameters such as maximum run speed, time, and maximal oxygen consumption (VO_{2max}) that can be used to describe aerobic endurance capacity, but may not be sufficient for detection of cardiovascular dysfunction. The exercise stimulus, particularly intensity and time of tests, are critical components that diverge from the GXT_h; thus limiting assessment of the cardiovascular function. It has been shown that large increments in intensity per stage are associated with a weaker relationship with VO_{2max}[17]. However, if a protocol is not intense enough or lasts longer than 12 minutes, exhaustion and performance are no longer the result of cardiopulmonary endpoints and instead become limited by variables such as orthopedic complications and specific muscle fatigue[17]. While most rodent assays increasing speed over a fixed inclination and result in tests lasting for time periods greater than 12 minutes[14, 20, 61-67], the GXT_h protocol simultaneous increases in speed and incline in stages over the course of 8-12 minutes[8].

By limiting commonly reported variables to VO_{2max}, run time, and maximum run

speed; critical cardio-metabolic measures such as anaerobic threshold (AT)[95], crossover (the shift from lipid to carbohydrate oxidation[96], and pre to posttest lactate concentrations ($Lactate_{delta}$)[97] are negated. These variables are measured by human GXT test but are rarely, if ever, reported in mouse exercise testing. This type of data provides valuable information regarding ability of the cardiac and pulmonary systems to deliver oxygen (O_2) during maximal and submaximal intensities[98]. Thus, failure to derive these values and other limitations with currently utilized exercise assays (reviewed in[24 23]) point out the need for a more efficacious and reliable standardized approach to test mouse cardiorespiratory function.

Here, we developed a standardized exercise assay (GXT_m), modeled after the human test and compared it to a PXT_m modeled off of protocols previously performed[14, 20, 61-67]. The testing in healthy mice and mouse models of cardiovascular dysfunction showed that variables derived from GXT_m but not from PXT accurately detect cardiovascular function.

3.2 Materials and Methods

Ethics Statement:

All animal experiments were performed in accordance with procedures approved by Ohio State University Laboratory Animal Resources (ULAR) committee and in accordance with the National Institutes of Health guidelines.

Animal subjects studied:

Animals were housed at The Ohio State University with 12hr light and dark cycles under standard conditions with unlimited access to food and water. C57Bl/6J, FVB/NJ (Jackson Laboratories, Bar Harbor, Maine), C57Bl/6J with high fat diet induced obesity (obese), and Calsequestrin 2 null (*Casq2*$^{-/-}$) (*Mus musculus*) male mice, approximately 4 months old, were used without randomization. Mice from each group were given ear tags and weight was recorded before all exercise sessions. The following groups were used: C57Bl/6J (*n* = 7), obese C57Bl/6J 9 (*n* = 11), FVB/NJ (*n* = 4), *Casq2*$^{-/-}$ (*n* = 4).

Animal acclimatization to treadmills:

Animals were first acclimated (**Table 3**) to treadmills (Metabolic Modular Treadmill; Columbus Instruments, Columbus, OH, USA) and then rested for one week prior to performing the GXT_m. Following performance on the GXT_m, animals

were again rested for one week before completing the PXT_m**.** All animals (*n* = 22, within-subjects design, GXT_m and PXT_m) underwent pre and post-test assays measuring blood lactate (LA) concentrations one hour prior to and immediately following exercise testing**.** During testing, maximum run speed (m/m), shock grid contact (seconds) and time until exhaustion (min) were manually recorded while the Oxymax computer software (Columbus Instruments, Columbus, OH, USA) collected gas concentrations and flow to calculate oxygen consumption (VO_2), carbon dioxide expiration (VCO_2), and respiratory exchange ratio (RER; the quotient of VCO_2/VO_2) from the treadmill. For exercise testing all testing was done on an open circuit indirect calorimetry treadmill (Metabolic Modular Treadmill, Columbus Instruments, Columbus, OH)[99]. All mice were acclimatized to treadmills the weeks prior to beginning testing for 3 sessions with 60 hours recovery between sessions. During the acclimation sessions, mice were placed in an unmoving treadmill for 3 minutes, after which the shock grid was activated (3 Hz and 1.5 mA). Following this the treadmill was engaged to a walking speed of 6 m/min for 5 minutes and progressively increased up to 12 m/min for a total duration of 12 minutes of exercise.

PXT_m Protocol:

Protocol was conducted as described in [93]. Mice were placed on the treadmill (0º incline entire experiment) and the shock grid was activated. The treadmill speeds were then increased until exhaustion as follows: (speed, duration) - (0 m/min, 5 min), (6 m/min, 5 min), (7, 8, 9, and 10 m/min, 30s each), (11m/min, 1 min), (12, 13, 14, and 15 m/min, 2 min each), and (+1 m/min, each 1 min thereafter). Exhaustion (endpoint for treadmill cessation) was defined as the point at which mice maintained continuous contact with the shock grid for 5 seconds. VO_{2max} was determined by the peak oxygen consumption reached during this test when the respiratory quotient (RER) was >1.0. Maximum running speed was defined as the treadmill speed at which VO_{2max} was achieved.

GXT_m Protocol:

Mice were placed on the treadmill at 0º incline and the shock grid was activated. The treadmill speeds were then increased until exhaustion as follows: (speed, duration, grade) - (0 m/min, 3 min, 0º), (6 m/min, 2 min, 0º), (9 m/min,2 minutes, 5º), (12m/min, 2 min, 10º), (15m/min, 2 min, 15º), (18, 21, 23, 24 m/min, 1 min, 15º), and (+1 m/min, each 1 min thereafter, 15º). Exhaustion (endpoint for treadmill cessation) was defined as the point at which mice maintained continuous contact with the shock grid for 5 seconds. VO_2max was determined

by the peak oxygen consumption reached during this test when the respiratory quotient (RER) was >1.0. Maximum running speed was defined as the treadmill speed at which VO_{2max} was achieved (**Table 1**).

Table 1. Summary of VO_{2max} testing protocols.

Description of the PXT_m. Stage	Speed (meter/min)	Elevation (% grade)	Duration (min)
1	6	0	5
2	7	0	0.5
3	8	0	0.5
4	9	0	0.5
5	10	0	0.5
6	11	0	1
7	12	0	2
8	13	0	2
9	14	0	2
10	15	0	2
11	16	0	1+
Description of the GXT_h. Stage	Speed (km/hr)	Elevation %grade	Duration (min)
1	2.7	10	3
2	4.0	12	3
3	5.4	14	3
4	6.7	16	3
5	8.0	18	3
6	8.8	20	3
7	9.6	22	3
Description of the GXT_m Stage	Speed (m/m)	Elevation %grade	Duration (min)
1	9	5	2
2	12	10	2
3	15	15	2
4	18	15	1
5	21	15	1
6	23	15	1
7	24+	15	1

Table 1: Summary of VO_{2max} testing protocols. Stages for the PXT_m (top), GXT_h (center), and GXT_m (bottom) are described.

Lactate assay:

A protocol was adopted[100] to measure venous lactate concentrations from the tail vein. During acclimation exercise sessions, mice were also acclimated to tail vein blood collection (3 pre acclimation session collections, and 3 post acclimation session collections). For the PXT_m and GXT_m; 1 hour prior to testing, 0.7μL of blood (via tail vein prick) was collected and placed for analysis on a handheld lactate meter (Lactate Plus; Nova Biomedical, Waltham, MA, USA). Within one minute of test completion, 0.7μL of blood was again collected and analyzed. For all testing, the same device was utilized to reduce variability.

Data Processing:

Prior to analysis, the dependent variables with the four genotypes (WT, Obese, *Casq2*$^{-/-}$, FVB/NJ) and two test types (GXT_m, PXT_m) were examined through IBM SPSS version 21 for accuracy of data entry, fit between their distributions, and the assumptions of multivariate analysis. Upon inspection of standardized scores, there were no univariate outliers. Therefore, no cases were removed from the data set. Tests of multivariate outliers (Mahalanobis distance values), assumptions of independence (review of plots of the residuals for each

dependent variable), and pairwise linearity (within-group scatterplots) was checked to assure all assumptions of normality for MANOVA were satisfied.

Obesity Mice, and Cardiac Mice on a set of outcome measures: relative VO_2 Max, absolute VO_2 Max, max speed, max time interval, delta VO_2, post lactate, delta lactate, % max VT, and % crossover max using three two-group MANOVAs (univateiate tests, The Bonferroni correction when applied to an alpha of .05 yielded an alpha level of .005).

Additionally, two three-group MANOVAs (Tukey HSD Multiple Comparisons, alpha = .004) were additionally performed to determine if there was a mean difference between genotypes for graded versus exercise tests on a set of outcome measures: relative VO_2 Max, absolute VO_2 Max, max speed, max time interval, delta VO_2, post lactate, delta lactate, % max VT, and % crossover max. Distance, total work, total power, VT, and crossover were also included as dependent variables for the MANOVA for the graded exercise tests.

3.3 Results

Development of a GXT exercise assay for mice.

Our initial goal was to develop a test similar to the human GXT_h and compare

parameters describing mouse performance during both the GXT_m and PXT_m. We used lean WT C57Bl/6J male mice as a control group, WT C57Bl/6J male mice with diet-induced obesity as an epigenetic metabolic model of cardiac deficiency, and calsequestrin deficient (*Casq2*$^{-/-}$) male mice as a genetic model with reported cardiac deficiency[61] (**Table 2)**. Within-subjects design for PXT_m and GXT_m exercise tests was used to reduce errors associated with individual differences. Following performance on the GXT_m, animals were rested for one week before completing the PXT_m (**Table 1).** All mice underwent pre and post-test LA assays one hour prior to and immediately following exercise**.**

We developed the GXT_m protocol taking into consideration that most PXT_m tests[13-15, 20, 58-67] increase in speed at a set incline over time (**Fig.1a, Table 1a**) until maximal exertion and respiratory exchange ratio (RER) ≥ 1.0 is achieved (**Table 4**). In the human GXT_h, (**Fig.1b, left, Table. 1**) there are simultaneous staged increases in speed and incline until the following conditions are met[80]: 1) maximal exertion, 2) achievement of respiratory exchange ratio (RER) ≥ 1.1, 3) a plateau or decrease following peak oxygen consumption (VO_{2max}), 4) a significant increase in pre- to post-test venous blood lactate concentrations (~8-10mmol/l),and 5) failure of heart rate to increase with increasing exercise intensity (**Table 4**). We developed a similar GXT exercise assay (**Fig.1b, right**

panel) in mice by have stages of simultaneous increases in speed and incline to achieve: 1) maximal exertion, 2) achievement of respiratory exchange ratio (RER) ≥ 1.0 3) a plateau or decrease following peak oxygen consumption (VO_{2max}), and 4) a significant increase in post test venous lactic acid concentrations. The end points of exercise tests are described in (**Table 4)**.

Oxygen consumption (VO_2) and carbon dioxide expiration (VCO_2) were two principal measures obtained in metabolic cages during the testing (**Fig.1c**). From these data we determined VO_{2max} and respiratory exchange ratio (RER; the quotient of (VCO_2/ VO_2) (**Fig.1d**); as well as, anaerobic threshold (AT) and fuel substrate (carbohydrate and lipid) oxidation (**Table 9**). Maximal exertion on the test was measured as time till exhaustion (minutes), as determined by ≥ 5 seconds of continuous contact with shock grid, and validated using biochemical measures of circulating lactic acid (LA) concentrations (mmol/L) (**Fig.1d**).

The GXT_m test derives measures similar to those reported in human testing

Next we demonstrated the ability of the GXT_m to generate measurements reported in human research. First we compared the results of healthy WT mice (male C57BL/6J, *n* = 7) performing PXT_m and GXT_m tests (**Fig 2**). Time until

exhaustion lasted for 20.2 to 29.1 minutes excluding warm-up with PXT_m (**Fig. 2, left panels**). The most commonly used test in humans, the Bruce protocol, elicits time till exhaustion at between 8-12 minutes in the general population[18, 80]. Similar to this $GXT_{h,}$ the GXT_m test achieved exhaustion between 8-12.5 minutes (excluding warm-up) in WT mice (**Fig. 2, right panels**). We then derive AT using VCO_2 data from each test (**Fig2a-b**). PXT_m and GXT_m both showed increases in VO_2 and VCO_2 during testing; however, in the majority of PXT_m single tests VCO_2 and VO_2 did not intersect at VO_{2max}, suggesting that true maximum was not achieved. In the $GXT_{m,}$ all single tests showed a clear intersection of VCO_2 and VO_2 (as demonstrated in **Fig. 2b, left)**. AT is the point at which there is a shift from aerobic to anaerobic metabolism, and additionally refers to an onset of metabolic acidosis during continuous exercise[101]. The standard method for determining AT is invasive[95] and not feasible during a VO_{2max} protocol preformed on a metabolic because mice are constrained in the closed circuit chamber and inaccessible for multiple blood draws while running (**Fig.1a**). Both single and group average GXT_m tests demonstrated that AT could be derived as an intersection of basal and abrupt increase in VCO_2 (**Fig. 2b**). VO_{2max} in GXT_m, but not in PXT_m, was additionally accompanied by increase in blood LA concentrations (data discussed in **Fig. 3**) and provided biochemical evidence of the transition from aerobic to anaerobic metabolism in only the GXT_m test.

RER kinetics showed a similar divergence in patterns depending on test utilized (**Fig. 2c, d**). AT value, as demonstrated by an abrupt increase in RER, was determined from $GXT_{m,}$ but not PXT_m test (**Fig. 2c, d**). With the GXT_h we also were able to determined the crossover point (the transition from fat to carbohydrate oxidation), a measure previously established in human testing[96]. A specific crossover point could not be determined by data from the PXT_m average (**Fig. 2e**) or from most single test data (not shown). However, each single GXT_m test and average test allowed for crossover determination (**Fig, 2f**). Of note, quantitative examination need to be performed in single test for measures generated from gas exchange (VO_2, VCO_2, RER, AT, crossover) whereas an averaged kinetics demonstrate the qualitative differences in measured derived in PXT_m and GXT_m tests.

The measures of GXT_m test detect impaired cardiorespiratory fitness.

Next, we compared the sensitivity of PXT_m and GXT_m tests for determination of cardiovascular fitness in mice. Maximal oxygen consumption (VO_{2max}) and VO_2 kinetics for WT lean, obese, and *Casq2*$^{-/-}$ is shown in **Fig. 3a-b**. With the PXT_m, VO_2 kinetics were similar between the *Casq2*$^{-/-}$ mice compared to the WT (**Fig.**

3a) and FVB/NJ control strains (**Tables 6-8**) but differed compared to WT using the GXT_m (**Fig. 3b**). Quantitatively, the PXT_m only resulted in a significant suppression of relative VO_{2max} in the obese dysfunctional group, but not the genetic *Casq2*$^{-/-}$ model compared to WT (MANOVA, Tukey HSD Multiple Comparisons, alpha = .007; **Fig 3c-d**). Similar to the differences observed in function v. dysfunctional animals' VO_2 kinetics with the GXT_m, the test also elicited significant suppression of relative VO_{2max} in the dysfunctional models compared to the WT control (alpha = .007, MANOVA, Tukey HSD Multiple Comparisons; p < .001, WT v. obese; p = .001; WT v. *Casq2*$^{-/-}$; p = .001 obese v. *Casq2*$^{-/-}$; **Fig 3c**). For all tests, there was an expected similar increase from basal VO_2 to VO_{2max} (VO_{2delta}) with the exception of VO_{2delta} values in WT v. obese using the PXT_m (alpha = .007, MANOVA, Tukey HSD Multiple Comparisons; p = .006, WT v. obese; **Fig. 3d**). However, this could have been observed as a result of the PXT_m eliciting a smaller VO_{2delta} in WT compared to the GXT_m (p < .05,Student's t-Test; **Fig. 3d**).

Similar to VO_{2max} data, only with the GXT_m was there a significant decrease in time until exhaustion in the *Casq2*$^{-/-}$ and obese mice (alpha = .007; MANOVA, Tukey HSD Multiple Comparisons; p = .006, *Casq2*$^{-/-}$; p = .001, obese; **Fig. 3e**). In the PXT_m no significant differences in time until exhaustion of healthy v.

dysfunctional models; indicating that that the test was not capable of determining which animals had cardiac insufficiency. Furthermore, *Casq2*$^{-/-}$ mice, a known model of cardiac insufficiency[61] ran longer than healthy WT controls (**Fig. 3e**). Of note, within each genotype's test-to-test performance, each PXT_m was significantly a longer test than the GXT_m.

The onset of exhaustion is validated by elevated post-test blood LA concentrations (~8-10mmol/L in humans) compared to baseline (LA_{delta} =LA_{post}-LA_{pre}) [18 23 24]. In mice we observed a significant increases in LA_{delta} using the GXT_m compared to the PXT_m (Student's t-Test; p = .05, test-to-test in healthy WT; p < .05, test-to-test in *Casq2*$^{-/-}$; **Fig 3f, Table 5**). With the GXT_m, *Casq2*$^{-/-}$ mice had significantly greater LA_{delta} compared to WT (Student's t-Test; p < .05, WT v. *Casq2*$^{-/-}$; **Fig 3e**); however, with the PXT_m, this parameter was decreased compared to controls. In humans with myocardial ischemia, a hallmark response to a GXT_h results in a significant increase in circulating blood LA concentrations compared to controls[102]. Thus, this response was replicated with the GXT_m in the genetic model of cardiac insufficiency (9.32 ± 1.53 mmol/L, *Casq2*$^{-/-}$ v. 6.63 ± 2.17 mmol/L; WT; Student's t-Test, alpha = .05; **Fig. 3f**). RER kinetics further indicated that PXT_m was not accurately assessing cardiovascular fitness; as *Casq2*$^{-/-}$ group was the best performing group qualitatively (**Fig 4a**). This

however was not seen with the GXT_m; as the WT performed the longest (**Fig 4a**) and ran the fastest. During testing, the point at which RER abruptly increases is known as AT[103]. The RER kinetics from all single GXT_m were capable of deriving this point; however, as single tests this was difficult to determine and some tests could not be used to determine AT (**Fig 4a,4b**).

Quantitatively, no significance was found between the point at which AT occurred in functional and dysfunctional mice using the PXT_m; however with the GXT_m, *Casq2*$^{-/-}$ mice also had significantly higher %AT compared to WT (alpha = .007, MANOVA, Tukey HSD Multiple Comparisons, p = .002; WT v. *Casq2*$^{-/-}$ during GXT_m; **Fig 4c**). Thus, similar to human research where AT is lower in patients with cardiac disease above functional class I[103], this was observed during the GXT_m in dysfunctional mice; and presented as occurring and then eliciting exhaustion in dysfunctional models (**Fig. 4c**). Interestingly, maximum run speed was only significantly reduced in both dysfunctional models during the GXT_m (alpha = .007, MANOVA, Tukey HSD Multiple Comparisons, p < .001; WT v. obese; p =.005; WT v. *Casq2*$^{-/-}$ during GXT_m; **Fig 4d**) and with the PXT_m, the *Casq2*$^{-/-}$ actually ran faster than the healthy WT controls (though not significantly).Together these results demonstrated that the GXT_m was superior for simultaneously eliciting an exhaustive effort and shift to anaerobic metabolism

as seen with the GXT_h[18, 24, 97, 102, 104].

Using the fuel substrate utilization during testing to classify degrees of cardiovascular dysfunction *in vivo*.

We determined values of carbohydrate or lipid oxidation during the both the PXT_m and GXT_m using information generated from RER values in each animal[96] (**Fig.5a-b, Supplementary Table 8,Fig.5a**). In humans, crossover occurs between 60-80% of aerobic power [96]. Similarly, we observed crossover in this range with all genotypes on the GXT_m, but occurred sooner in the *Casq2*$^{-/-}$ compared to WT controls (student's T-test, $p < .05$; WT v. *Casq2*$^{-/-}$ **Fig.5g**). Time to 100% carbohydrate oxidation during testing an rate of carbohydrate oxidation after crossover, were significantly shorter in dysfunctional animal; (Time until 100% carb oxidation, student's T-test, $p < .05$; WT v. *Casq2*$^{-/-}$, $p < .05$; WT v. obese,**Fig.5h**; rate of carbohydrate oxidation after crossover, student's T-test, $p < .05$; WT v. *Casq2*$^{-/-}$, $p < .05$; WT v. obese,**Fig.5i**) indicating the ability of the GXT_m to utilized substrate utilization to identify dysfunction.

Figure 1| Fundamentals of exercise testing in mice and men.

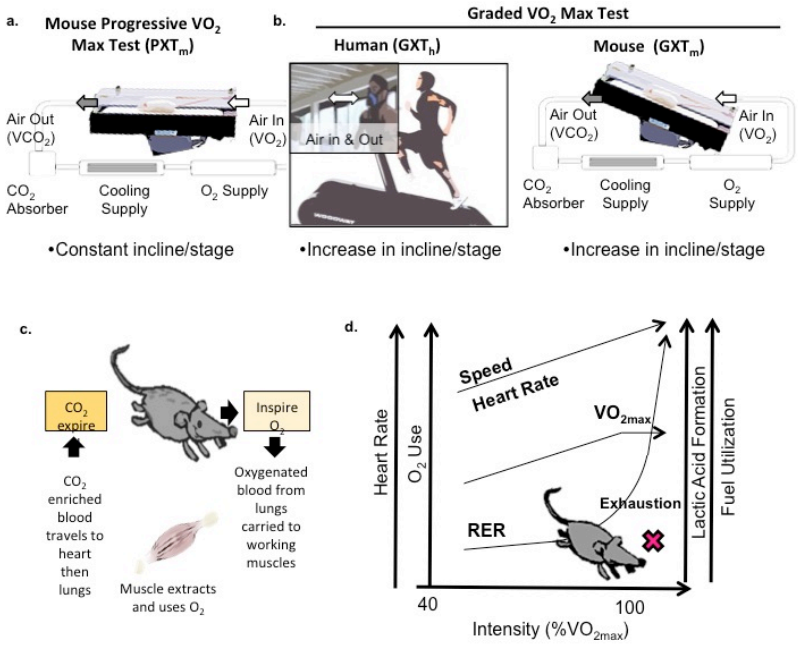
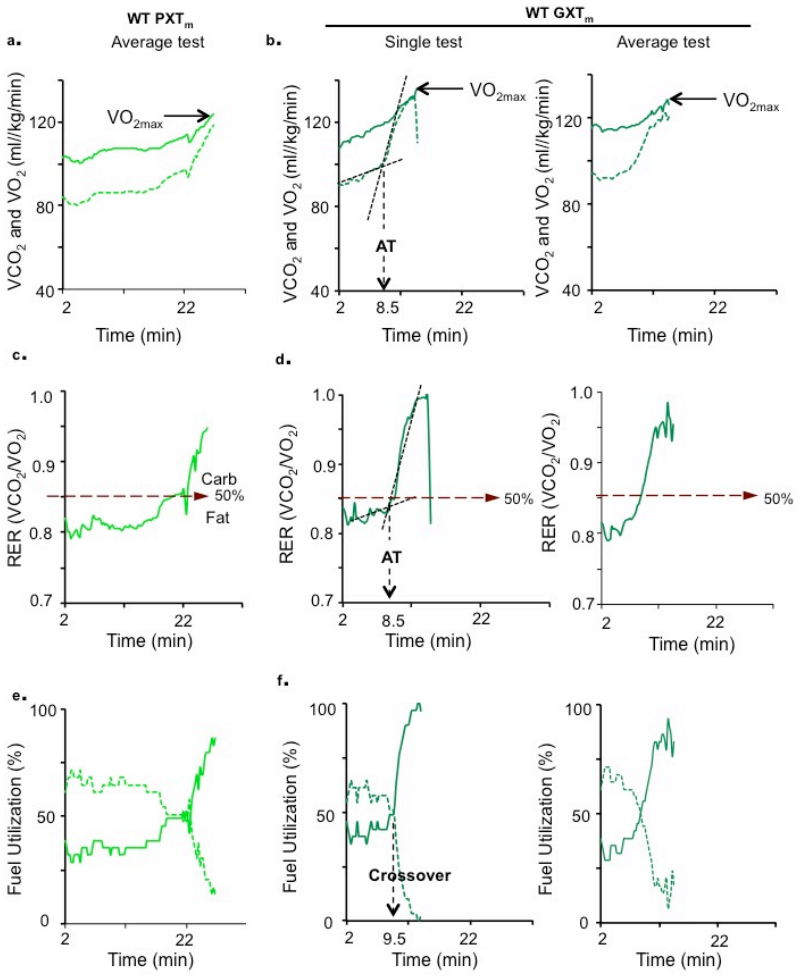


Figure 1| Fundamentals of exercise testing in mice and men. **(a)** The PXT_m (left) maintains fixed inclination (0˚) while speed increases until the test is terminated (**Table1**). Destining on mice is done in a treadmill chamber that acts as an open circuit indirect calorimeter and thus allows for collection of VO_2, VCO_2 production, and RER calculation. **(b)** With the GXT_h (middle) and GXT_m (right), speed and incline simultaneously increase as stages progress (**Table1**). The human test also uses indirect calorimetry using a metabolic cart to retrieve measures of VO_2 and VCO_2. **(c)** During exercise, animals need to consume O_2 so that oxygenated blood can be circulated to working muscles. Mitochondria in muscles use this O_2 to generate ATP and fuel contractions during aerobic exercise. Once O_2 is extracted at the muscle, deoxygenated blood then travels to the heart then lungs, and CO_2 is expired. **(d)** During the exercise assay, there should be an increase in speed and heart rate over the course of the test, an increase and then peak or plateau of VO_2, and linear increase which becomes interrupted by an abrupt increase in RER indicating AT as the test progresses till exhaustion. As this intensity increases, lactic acid builds up. There is also a shift from fat to carbohydrate (carb) oxidation.

Figure 2| Differences in analysis and interpretation capabilities of utilizing the PXT_m v. GXT_m demonstrated with data from WT mice

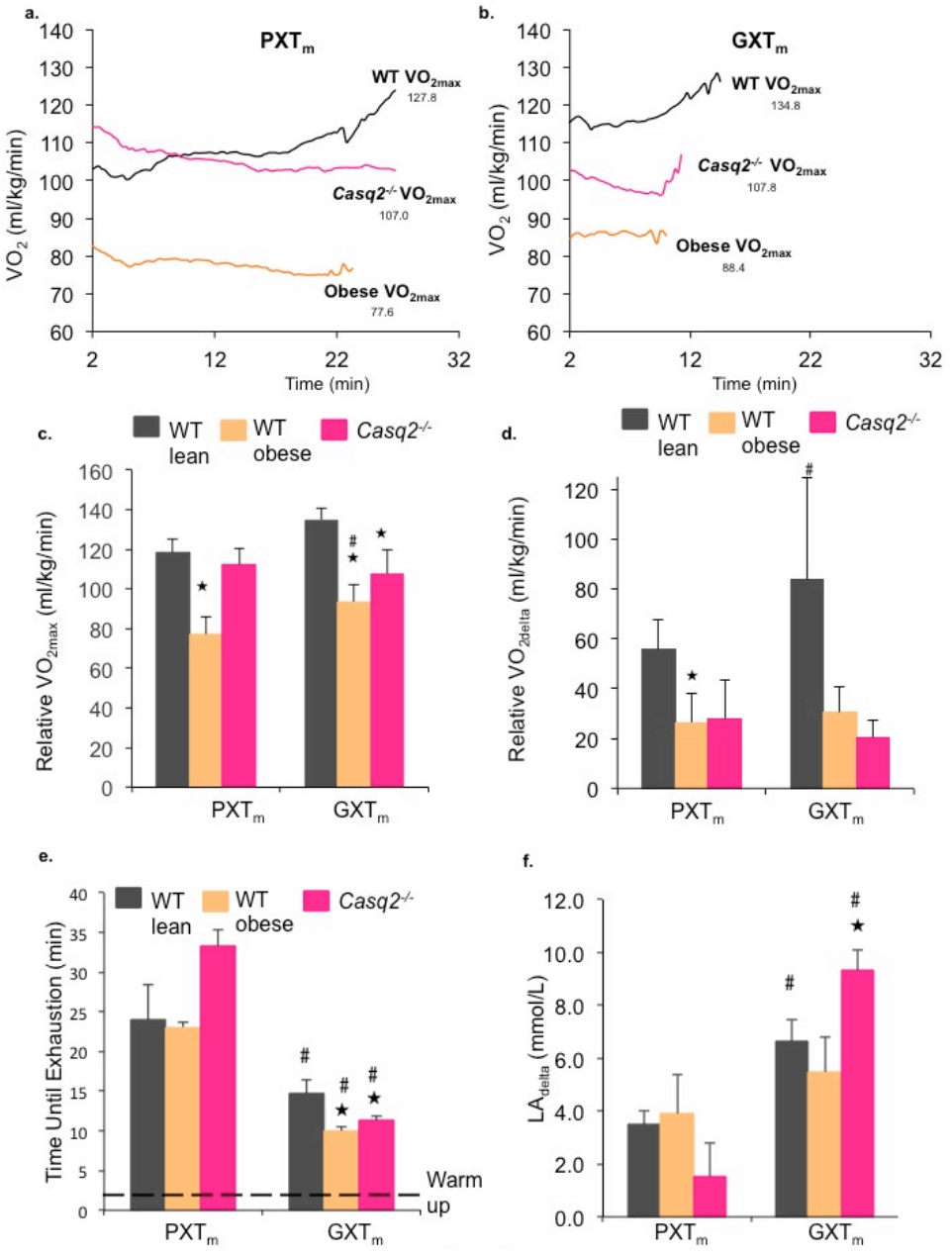


(Figure 2; continued)

(Figure 2; continued)

Figure 2| Differences in analysis and interpretation capabilities of utilizing the PXT_m v. GXT_m demonstrated with data from WT mice. **(a)** VCO_2 (light green, dotted) and VO_2 (light green, solid) values during PXT_m test in WT mice. VO_{2max} (ml/kg/min) is indicated by black arrow and is representative of maximal VO_2 achieved during testing. **(b)** VCO_2 (dark green, dotted) VO_2 (dark green, solid) and from a single WT C57BL/6J male mouse (left) and group average (right) were plotted against time during GXT_m. Black arrow indicated VO_{2max}. **(c)** RER, the quotient of VCO2/VO2 is plotted against time to represent fuel substrate utilization on the same WT animals. An RER of .85 indicates 50% carbohydrate and 50%fat oxidation as demonstrated in the PXT_m and is displayed in current and **(d)** subsequent GXT_m single test (left) and group (right) graphs by red dotted line with arrow. In the single GXT_m test (left) the point in which there is an abrupt increase in RER is known as AT and indicated with by black arrow and occurs at the intersection of the two dotted black lines. **(e)** Fuel utilization, as represented by percent carbohydrate (green, solid) and fat (green, dotted) oxidation, determined from RER values (**Table 9**), are plotted against time in the PXT_m,**(f)** GXT_m single test (left) and group (right). In single test, black arrow indicates the point (time) in the test (09:30) in which there is a shift from predominant lipid oxidation to predominate carbohydrate oxidation, and is identified as "crossover." Values represent mean unless noted; *n* = 7 mice. **Abbreviations:** Respiratory exchange ratio, **RER**; anaerobic threshold, **AT**; volume of carbon dioxide exhaled, **VCO_2**; volume of oxygen consumed, **VO_2**; maximal oxygen consumption during test, **VO_{2max}**.

Figure 3| VO_2 information generated utilizing the PXT_m v. GXT_m in functional and dysfunctional animals



(Figure 3; continued)

(Figure 3; continued)

Figure 3| VO_2 information generated utilizing the PXT_m v. GXT_m in functional and dysfunctional animals. **(a)** VO_2 values from the PXT_m and **(b)** GXT_m in the same WT (black), *Casq2*$^{-/-}$ (red), and Obese (orange) are plotted against time. VO_{2max} (average) is indicated per each testing group in bold. **(c)** Relative VO_{2max} values, **(d)** VO_{2delta} **(e)** time until exhaustion (min) as defined as 5 seconds of continuous contact with shock grid and **(f)** LA_{delta} (mmol/L) from the PXT_m (left) and GXT_m (right) are shown in WT (grey), Obese (orange) and *Casq2*$^{-/-}$ mice. Star indicates significance at the alpha = .007 level (MANOVA, multiple comparisons Tukey HSD for the PXT_m and GXT_m) and hash indicates significant difference at the alpha = .05 level between tests for the same genotype (Student's t-Test). Line graph values represent mean and bar graphs represent mean ± SD for all but lactate which ± s.e.m; *n* = 22. **Abbreviations:** oxygen consumption, **VO_2**; relative maximal oxygen consumption, **relative VO_{2max}**; change from baseline to maximal oxygen consumption, **VO_{2delta}**; change from pre to post test lactate concentration, **LA_{delta}**.

Figure 4| RER information generated utilizing the PXT_m v. GXT_m in functional and dysfunctional animals

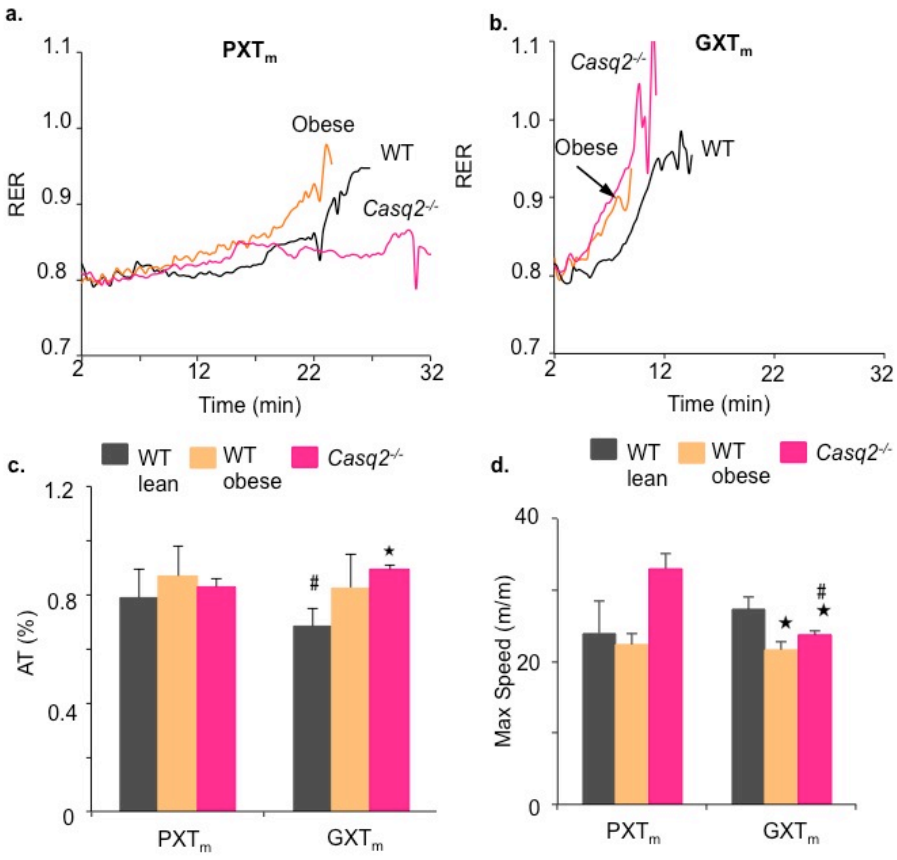
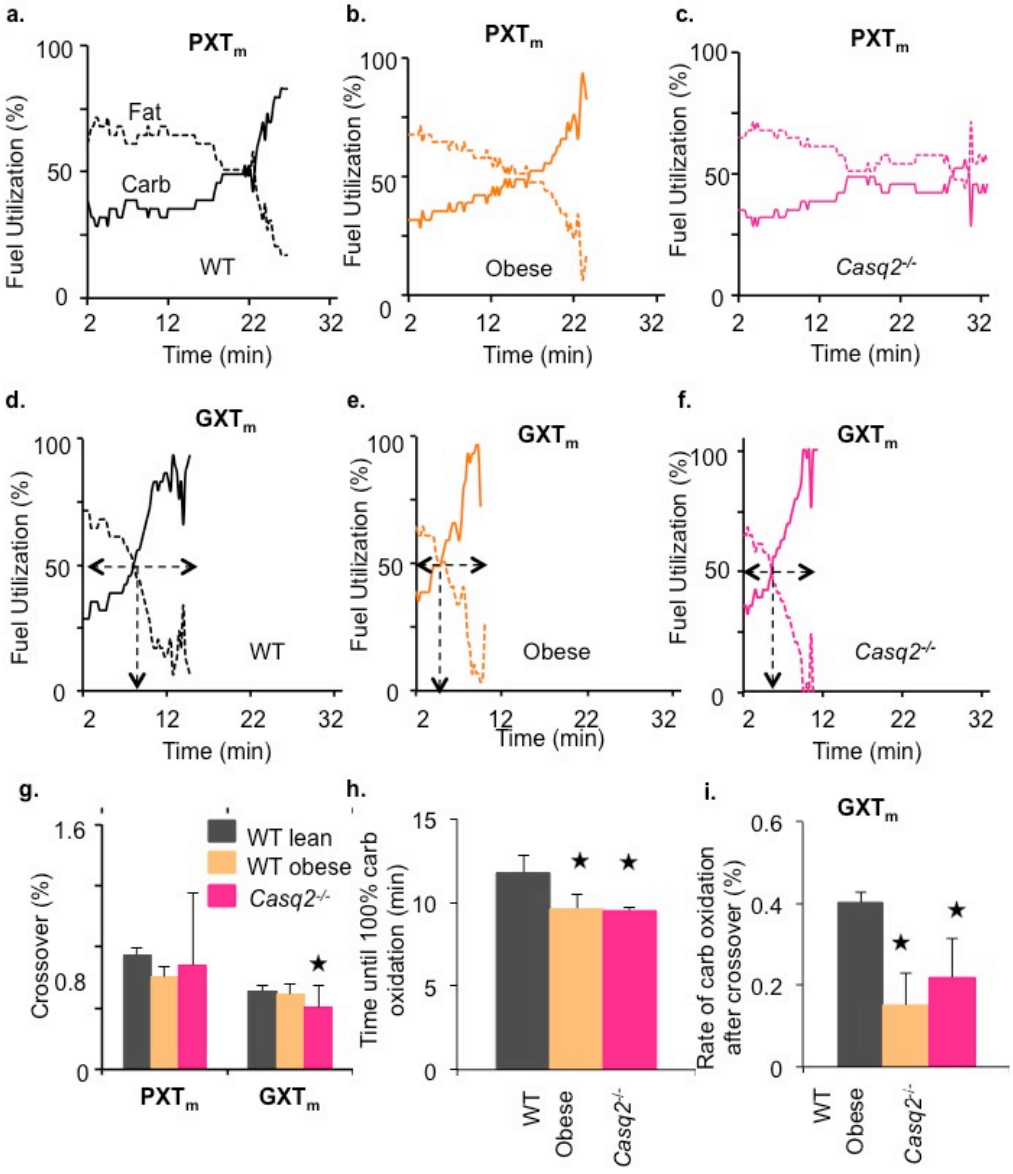


Figure 4| RER information generated utilizing the PXT_m v. GXT_m in functional and dysfunctional animals. **(a)** RER values are plotted over time during the PXT_m and **(b)** GXT_m in the WT (black), obese (orange) and *Casq2*$^{-/-}$ (red) mice. **(c)** percent of test at which AT (the abrupt increase in RER) occurred and **(d)** maximum speed achieved on test (m/m), during PXT_m (left) and GXT_m (right) in WT (grey), obese (orange) and *Casq2*$^{-/-}$ (red) mice. Star indicates significance at the alpha = .007 level (MANOVA, multiple comparisons Tukey HSD for the PXT_m and GXT_m) and hash indicates significant difference at the alpha = .05 level between tests for the same genotype (Student's t-Test). Line graph values represent mean and bar graphs represent mean ± SD; *n* = 22.
Abbreviations: Respiratory exchange ratio, **RER**; anaerobic threshold, **AT.**

Figure 5| Differences in analyses and interpretation of carbohydrate and fat utilization data utilizing PXT_m v. GXT_m in functional and dysfunctional animals.



(Figure 5; continued)

(Figure 5; continued)

Figure 5| Differences in analyses and interpretation of carbohydrate and fat utilization data utilizing PXT_m v. GXT_m in functional and dysfunctional animals. (**a-c)** RER is transformed using carbohydrate and fat for nonprotein RER values per liter of oxygen (**Table 9**) and plotted against time during the PXT_m and **(d-f)** GXT_m tests in WT (black), obese (orange), and *Casq2*$^{-/-}$ (pink) mice. The point at which carbohydrate (solid) and lipid (dotted) oxidation cross is deemed the crossover point and is indicated by arrow and quantified as **(g)** percent of the test at which the crossover point was achieved, **(h)** time until 100% carbohydrate oxidation and **(i)** rate of carbohydrate oxidation after crossover as determined by dividing time after crossover to 100% carbohydrate oxidation by time of test in WT (black), obese (orange), and *Casq2*$^{-/-}$ (pink) mice. For **5g**, star indicates significance at the alpha = .007 level (MANOVA, multiple comparisons Tukey HSD for the PXT_m and GXT_m) and hash indicates significance at the alpha = .005 (MANOVA; univariate tests, Bonferroni correction) and for **5h-i**, star indicates significance at the alpha = .05 level (student's T-test for the PXT_m and GXT_m between genotypes). Line graph values represent mean and bar graphs represent mean ± SD; n = 22.

Abbreviations: percent of test where crossover point occurs, **%crossover**.

3.4 Discussion

For over 50 years, exercise testing has served as an established and validated method for diagnostic and prognostic assessment of cardiovascular function in the clinical setting[80]. Physicians and exercise physiologists value the use of the GXT_h, as it is capable of inducing physiological stress[105] to the cardiopulmonary system in a controlled environment while simultaneously monitoring myocardial oxygen demands[106], biochemical[107] and metabolic[96] responses. Furthermore, it

is a validated method of evaluating the status of patients with cardiovascular and pulmonary disease[17].

Taking the opposite approach: Translating findings from man to mouse

Established GXT_hs involve gradual increases in work output over multi-stage increases in speed and incline[79](**Table 1**). When generating the GXT_m, we applied the same principle concept of simultaneously staged increases in speed and inclination but and made slight adjustments to stage length and intensity to account for differences between the physiologies of mouse v. man during exercise[12]. By modeling the GXT_m off of the GXT_h and utilizing similar criteria for a positive test (**Table 4**), test performance of the mice was no longer susceptible to limitations by specific muscle fatigue as it had been on the longer test. Instead, the test was specific to evaluating the ability of the cardiovascular system to supply O_2 to the muscles and of the pulmonary system to clear CO_2 from the blood[17].

Addressing the limitations

Software from metabolic treadmills commonly used by researchers are open circuit indirect calorimeters and contain O_2 and CO_2 sensors that allow for the collection of gas exchange data (CO_2, VO_2, RER). However, without the ability to

record ventilation during testing[12], the amount of information deducted from maximal exercise testing can be considered limited. "Oxygen recovery tests" have been used to validate gas measurements recorded using open-circuit direct calorimeter treadmill chambers[12]. Thus, the loss of information retrieved from the testing can be minimized so long as the testing method follows established GXT_h considerations: (1) modest stage to stage increases in energy requirements (2) allowance for achievement of steady state before increasing the stage (3) a testing duration greater than 6 minutes and (4) no longer than 12 minutes[17, 108]. In human research, the relationship between VO_2 and work rate diminishes in tests lasting less than 6 minutes or greater than 12 minutes[17]. Additionally, others have shown that tests lasting over 12 minutes provide data that is impacted by skeletal muscle fatigue and orthopedic issues[17]. The PXT_m is composed of a large volume of submaximal work. During this exercise intensity, there is a decreased demand for oxygen and a reduction in redistribution of blood from inactive to active tissues. As a result, there is a decrease in cardiac output, ventilation, and VO_2 due to the resulting reductions in muscle mass recruitment[20]. In the PXT_m, this type of scenario occurs, as the cardiovascular system is not maximally stressed until later stages of the test and can be considered too long to specifically stress the cardiorespiratory system and its ability to withstand metabolic stress.

Anaerobic threshold as a diagnostic

Limitations of having a longer test can be problematic if a researcher wants to report values beyond maximum run speed or duration to determine the cardiovascular phenotype of a mouse. This was observed in our data, as some tests preformed using the PXT_m were incapable of producing a point which we could determine to be AT. Classically AT can be identified by a nonlinear increase in minute ventilation [105]. However, given that this is not feasible for most researchers to calculate in mouse models during exercise[12], we chose to use the abrupt exponential increase in RER[101, 103] or a nonlinear increase VCO_2[103] to determine the point at which AT occurred in both WT and dysfunctional models. Clinically, AT has been used in patients with cardiorespiratory disease to assess exercise tolerance[101]; however, the ability to derive AT from an exercise test has applications such as evaluating endurance performance, exercise prescription, and determining the effects of drugs on exercise tolerance (reviewed in[109]). Thus, the value of being able to derive this value from the exercise assay in a mouse model, as shown with the GXT_m, highlights novel diagnostics that can be derived from this assay.

Fuel utilization as a quantitative measure of cardiovascular function during exercise test.

The GXT_h was not just capable of predicting AT based off of RER values, but it was also capable of determining the crossover from predominate lipid to carbohydrate oxidation during testing. This shift in fuel substrate utilization, (known as the crossover concept[77]), demonstrates that as relative VO_2 and power output increase, there is a switch from the predominance of lipid oxidation to an increasing dependence on muscle glycogen and blood glucose substrates[77]. The concept, which had been well established with methods such as radio-tracers, tissue metabolite sampling, stable isotopes, and indirect calorimetry in mammals and man[78] (reviewed in[77]), is the basis of how we developed metabolic parameters to determine dysfunction based on carbohydrate oxidation. With the GXT_m, our use of glycogen and glucose oxidation as exponential functions of exercise intensity[77] allowed parameters which could predict cardiovascular function (**Fig. 5g-i**). It should be noted, as demonstrated in the data of a single WT mouse, that AT and crossover do not occur simultaneously, potentially in part due to pyruvate dehydrogenase (PDH) mediated LA accumulation and aerobic substrate oxidation[79]. In working muscles, transformation of the pyruvate dehydrogenase complex (PDHc) to the active form (PDHa) is complete at approximately 80%VO_{2max}[80]; however crossover occurs at approximately 65% percent of VO_{2max} [58]. With the GXT_h, both

crossover and AT were found around these approximations; with crossover occurring at 62-75% and AT occurring between 68-87% (**Fig 5g**).

Dysfunctional animals, despite experiencing no significant difference in %crossover compared to WT animals, had AT occur at higher percentages of their VO_{2max} (as seen with cardiac patients in human studies[66]). As exercise intensity reaches near maximum, glycolytic flux is accompanied by LA formation and disposal, and a shift to anaerobic metabolism occurs. In dysfunctional animals performing the GXT_m, this shift is shortly followed by test termination, and represented as a steep linear rate of carbohydrate oxidation and failure to maintain work output at maximum rates of carbohydrate oxidation.

Standardized methods for the functional assessment of cardiovascular function in mouse models

Without a gold standard *in vivo* exercise assay, reported data becomes both unreliable and difficult to reproduce between researchers. Without considering (1) the components human researchers have studied to create their testing, (2) the limitations of the test, and (3) alternative values that can be generated from gas exchange data; researchers are limited to their phenotypic descriptions. However, with the appropriate considerations, a test such as the GXT_m can serve

as a noninvasive functional *in vivo* assay to assess the cardiovascular phenotype of a mouse and the effects of various treatments and therapeutics. While cardiac challenges using echocardiograph (EKG)[113] and cardiac magnetic resonance imaging ($_c$MRI)[114] are popularized protocols to stress and test the cardiovascular system in mouse models, they are expensive, invasive, and require animals to be euthanized. In human research, the GXT_h shows a 73% predictive accuracy when compared to Dobutamine echocardiography (86%)[80]. However, animal testing, unlike human testing, classically calls for euthanasia. This prevents animal heart rate from achieving the true physiological response to the reagent. Consequently, cardiac output, a measure of blood being pumped by the heart per minute, is compromised. Unlike these invasive procedures, exercise assays can elicit a 2-fold in increase in cardiac output[115] and measure cardiac output without invasive limitations or euthanasia.

Appropriate exercise testing and prescription clearly has its place in human physiology and accordingly, considerations in prescription and testing of mice can carry similar weight. If research done on mice is to elucidate mechanisms of disease and therapies, then it is critical to apply the appropriate stimuli to ensure that the results observed are achieved through the appropriate modality. Here we established a proof-of-principle experiment showing that we could integrate

fundamental considerations of a GXT_h to generate a sensitive, superior, noninvasive, and cost effective method to determine the cardiovascular, metabolic, and pulmonary phenotype of various mouse models. Future work implementing this method will allow for standardization amongst researchers and improved abilities to translate exercise related findings from mouse to man.

3.5 Supplementary information

Table 2. Baseline Genotype Parameters

Genotype	Weight (g)	Basal VO_2 (ml/kg/min)	Basal LA (mmol/L)
WT C57Bl/6J	28.15 ± 1.46	73.14 ± 11.56	2.44 ± 1.22
Obese C57Bl/6J	45.42 ± 2.66*	55.24 ± 11.84*	2.89 ± 1.08
Casq2$^{-/-}$	25.72 ± 0.87*	86.89 ± 7.32+	3.56 ± 1.79
WT FVB/NJ	29.23 ± 1.84	74.10 ± 9.20	2.03 ± 0.86

Table 2. Baseline Genotype Parameters. Baseline parameters for mice used in study are listed (n = 22). Plus indicates significance between tests (p < .05, Student's t-Test) and star indicates significance between genotypes (alpha = .007; MANOVA, multiple comparisons Tukey HSD).

Table 3. Animal Acclimation to Treadmill Protocol.

Stage	Speed (meter/min)	Elevation (% grade)	Duration (min)
1	0	0	3
2	6	0	5
3	9	0	2
4	12	0	2

Table 3. Animal Acclimation to Treadmill Protocol. Animals were acclimated to treadmills using the following protocols for 3 sessions prior to performing maximal exercise tests.

Table 4: End points from the PXT_m, GXT_h[80], and GXT_m.

Test End Points	RER	VO_2 Plateau	LA Conc. (mmol/L)	Exertion
PXT_m	≥ 1.0	Measurement not required	Measurement not required	5+ seconds of continual contact with shock grid
GXT_h	≥ 1.1	Plateau/decline O_2 uptake with increasing workload	Significant increase in venous LA conc. Post test (~8-10mmol/L)	RPE ≥ 9 out of 10
GXT_m	≥ 1.0	Plateau/decline O_2 uptake with increasing workload	Significant increase in venous LA conc. Post test (~8-10mmol/L)	5+ seconds of continual contact with shock grid

Table 4: End points from the PXT_m, GXT_h[80], and GXT_m. End points for the GXT_h were derived from ACSM guidelines[80], PXT_m end points were generated based off of criteria previously, and commonly reported, and GXT_m end points were developed to best replicate end points in human testing.

Table 5. End points from the GXT_m and PXT_m in functional and dysfunctional animals

Genotype	Relative VO_{2max} (ml/kg/min)	Maximum Run Speed (m/m)	Time Till Exhaustion (min)	LA_{delta} (mmol/L)
PXT_m				
WT	127.06 ± 6.76	26.50 ± 4.43	26.75 ± 4.48	4.08 ± 1.38
Obese	77.64 ± 8.30*	22.75 ± 1.75	23.34 ± 1.63	5.15 ± 3.19
Casq2$^{-/-}$	107.00 ± 8.66	32.33 ± 2.08	32.82 ± 1.66	2.50 ± 2.52
GXT_m				
WT	134.84 ± 5.80	27.29 ± 1.70	14.68 ± 1.78	6.63 ± 2.17
Obese	88.52 ± 7.17*	21.64 ± 2.06*	10.05 ± 0.84*	4.51 ± 3.08
Casq2$^{-/-}$	107.77 ± 11.80*	23.75 ± 0.50*	11.31 ± 0.55*	9.32 ± 1.53*

Table 5. End points from the GXT_m and PXT_m in functional and dysfunctional animals. Based on observed means. Star indicates a significant difference between genotypes for either the PXT_m or GXT_m at the alpha = .007 level (MANOVA, multiple comparisons Tukey HSD, *n* = 22).

Table 6. End points from the GXT_m and PXT_m in WT C57Bl/6J and FVB/NJ animals.

Genotype	Relative VO_{2max} (ml/kg/min)	Maximum Run Speed (m/m)	Time Till Exhaustion (min)	LA_{delta} (mmol/L)
PXT_m				
WT	127.06 ± 6.76	26.50 ± 4.43	26.75 ± 4.48	4.08 ± 1.38
FVB/NJ	119.73 ± 6.35	39.50 ± 3.79	39.89 ± 3.77*	3.23 ± 1.39*
GXT_m				
WT	134.84 ± 5.80	27.29 ± 1.70	14.68 ± 1.78	6.63 ± 2.17
FVBN/J	119.13 ± 9.56*	29.25 ± 2.5	16.56 ± 2.73	3.23 ± 1.39

Table 6. End points from the GXT_m and PXT_m in WT C57Bl/6J and FVB/NJ animals. Based on observed means. Star indicates significance at the alpha = .05 level in WT v. FVB/NJ on a given test (Student's t-Test, *n* = 11).

Table 7. End points from the GXT_m and PXT_m in FVB/NJ v. *Casq2*$^{-/-}$ animals.

Genotype	Relative VO_{2max} (ml/kg/min)	Maximum Run Speed (m/m)	Time Till Exhaustion (min)	LA_{delta} (mmol/L)
PXT_m				
FVB/NJ	119.73 ± 6.35	39.50 ± 3.79	39.89 ± 3.77	3.23 ± 1.39
Casq2$^{-/-}$	107.00 ± 8.66	32.33 ± 2.08*	32.82 ± 1.66*	2.50 ± 2.52
GXT_m				
FVBN/J	119.13 ± 9.56	29.25 ± 2.5	16.56 ± 2.73	3.23 ± 1.39
Casq2$^{-/-}$	107.77 ± 11.80	23.75 ± 0.50*	11.31 ± 0.55*	9.32 ± 1.53*

Table 7. End points from the GXT_m and PXT_m in FVB/NJ v. *Casq2*$^{-/-}$ animals. Based on observed means. Star indicates significance at the alpha = .05 level in FVB/NJ v. *Casq2*$^{-/-}$ on a given test (Student's t-Test, *n* = 8).

Table 8. Absolute end points from functional and dysfunctional mice.

Genotype	Absolute VO_{2max} (ml/min)	Post LA (mmol/L)
PXT_m		
WT	4.59 ± 0.25	6.53 ± 1.49
Obese	1.70 ± 0.16*	7.31 ± 2.18
Casq2$^{-/-}$	4.19 ± 0.49	7.60 ± 1.04
GXT_m		
WT	4.76 ± 0.29	9.06 ± 2.20
Obese	1.97 ± 0.22*+	8.12 ± 2.78
Casq2$^{-/-}$	4.19 ± 0.56	10.50 ± 2.05

Table 8. Absolute end points from functional and dysfunctional mice. Based on observed means. Plus indicates is significance at the .005 level between test per genotype (ANOVA, Bonferroni correction, univariate results for genotype). Star indicates significance between groups on each protocol at the alpha =.004 level (MANOVA, Tukey HSD Multiple Comparisons, Univariate results for test); *n* = 22.

Table 9. Percentage of carbohydrate and fat oxidation for nonprotein in RER values for each liter of oxygen utilized[116, 117]

RER	%CHO	%FAT	RER	%CHO	%FAT
.7	0.0	100.0	.86	52.4	47.6
.71	1.4	98.6	.87	55.8	44.2
.72	4.8	95.2	.88	59.2	40.8
.73	8.2	91.8	.89	62.6	37.4
.74	11.6	88.4	.90	66.0	34.0
.75	15.0	85.0	.91	69.4	30.6
.76	18.4	81.6	.92	72.8	27.2
.77	21.8	78.2	.93	76.2	23.8
.78	25.2	74.8	.94	79.6	20.4
.79	28.6	71.4	.95	83.0	17.0
.80	32.0	68.0	.96	86.4	13.6
.81	35.4	64.6	.97	89.8	10.2
.82	38.8	61.2	.98	93.2	6.8
.83	42.2	57.8	.99	96.6	3.4
.84	45.6	54.4	1.0	100.0	0.0
.85	49.0	51.0			

Table 9. Percentage of carbohydrate and fat oxidation for nonprotein in RER values for each liter of oxygen utilized[116, 117]. Analysis of the oxidation of mixtures of carbohydrate and fat (derived from Lusk[116]). Formulas which Oxymax software utilizes, as well as my fuel substrate charts, are based off of the study of metabolism in dog and biochemical data has shown inconsistency in table derivations[118]. Calculations were derived using direct animal (dog) calorimetry, radioactive isotope labeling of isotopes 12 and 13 of carbon, and urinary nitrogen excretion measurements.

Supplementary Note 1: Using the FVB/NJ as a control for the *Casq2*$^{-/-}$ model.

The FVB/NJ mouse, compared to the C57BL/6J has been shown to have significantly superior performance on endurance exercise testing in measurements for duration, distance and work performance[119 120]. To account for this we performed all tests on the FVN/NJ mouse (**Table 6-8**) and found its fitness was superior to even the WT (Student's t-Test, $p = 0.05$) with increased max speed and time till exhaustion on the PXT_m. Interestingly, only the GXT_m, was able to show that the FVB/NJ had increases in relative VO_{2max}, VO_{2delta}, post LA, and LA_{delta}. This indicated the GXT_m had superior sensitivity compared to the PXT_m, as a result to specific stress to the cardiovascular system without long duration capable of inducing additional fatigue to the musculoskeletal system. When comparing the performance of the *Casq2*$^{-/-}$ to the FVB/NJ on the GXT_m, there was a significant difference in max speed, time until exhaustion, VO_{2delta} (student's t-Test, $p < .05$) and with the PXT_m there was a difference in speed, VO_{2delta}, and post LA (student's t-Test, $p = .05$) indicating that there was no difference in time until exhaustion. For the FVB/NJ in a test to test comparison, the PXT_m resulting in a significantly lower LA_{delta} (student's t-Test, $p < .01$; 3.22 ± 1.38 mmol/L, PXT_m; 6.96 ± 1.35 mmol/L, GXT_m, **Table 6-8**) indicating that even

with superior fitness, the GXT_m was superior in eliciting a significant increase in LA concentrations; which is an end point for a positive VO_{2max} test in humans [18].

Supplementary Note 2: Gas exchange equations.

All treadmill testing was done in a metabolic modulator treadmill (Columbus Instruments, Columbus, OH, USA). This treadmill (24" exercise belt, speeds from 0 m/m to 99.9m/m, inclination -10˚ to 25˚, adjustable shock grid from 0.35mA to 1.5mA) is enclosed in an air-tight isolated chamber (29"L x 27"W x 17.5"H) allowing it to function as an open circuit indirect calorimeter. As the result of functioning as an indirect calorimeter, oxygen consumption (VO_2) and carbon dioxide expiration (VCO_2) can be calculated by the Oxymax software. This software is dependent on accurate measurements of gas concentrations and flow, and thus needs to be calibrated prior to all experiments involving gas exchange assessments.

To make these calculations during testing the software collects values of either the mass of air at chamber input per unit of time (Vi) or the mass of air at chamber output per unit of time (Vo) and then predicts the alternate flow (under

the assumption that N2 is equal in the input and output portion of the chambers, and does not take part in respiratory gas exchange):

Vi = Mass of air at chamber input per unit time

O_2i = Oxygen fraction in Vi

CO_2i = Oxygen fraction in Vi

Vo = Mass of air at chamber output per unit time

O_2o = Carbon Dioxide fraction in Vo

CO_2o = Carbon Dioxide fraction in Vo

From those values VO_2 and VCO_2 are calculated:

$$VO_2 = ViO_2i\ i\text{-}VoO_2o$$

$$VCO_2 = VoCO_2o\ \text{-}ViCO_2i$$

From VO_2 and VCO_2 values collected, the software can then determine respiratory exchange ratio (RER):

$$RER = VCO_2 / VO_2$$

From RER are between 0.7 and 1.0 carbohydrate and fat oxidation per liter of oxygen used can be calculated (**Table 9**)[121] as can the percent of carbohydrates and fat oxidized per minute.

$$CHO\ (g/min) = -3.226 * VO_2(L/min) + 4.585*VCO_2(L/min)\ ^{118}$$

$$FAT\ (g/min) = 1.695 * VO_2(L/min) - 1.701* VCO_2(L/min)\ ^{118}$$

Table 10:

RER	Heat/liter O_2 (kcal)	RER	Heat/liter O_2 (kcal)
.707	4.6862	.90	4.9226
.75	4.7387	.95	4.9847
.80	4.8008	1.0	5.0468
.85	4.8605		

Table 10: RER and derived heat values. For RER values between 0.7 and 1.0, 4.686 to 5.047 Kcal/Liter O_2 (Heat) is available (from [122]).

To calculate the caloric value, the following formula can then be applied using RER values:

$$CV\ (kcal/liter\ of\ O_2) = 3.815 + 1.232 \times RER$$

Calculating the caloric value allows for the derivation of energy expenditure (heat) of a mouse during exercise:

$$Heat\ (kcal/hour) = CV(kcal/liter\ of\ O_2) \times VO_2\ (ml/kg/hr)$$

*Note, we reported VO_2 as ml/kg/hr

Additionally, VO2 values can be used to derive metabolic equivalents (METs)

$$MET = VO_2/kg \div 3.5$$

VO_{2max} is critical to determine, as workload can then be quantified as METS (metabolic equivalents), and adapted from ACSM recommendations for general and special populations to prolonged exercise experiments in mice. When METS are used in combination with various other metrics like AT, more specific recommendations can be made[1,123,80]. METs can be used to prescribe intensities in long-term exercise experiments. Considering exercise intensity for experiments using exercise in mouse models has its place. In some models with cardiovascular and/or skeletal muscle limitations, exercise that is too great in intensity may elicit maladaptation, as the stress is to great for the organism to overcome. In other scenarios, intensity might not enough to elicit an adaptation. Exercise, which is a stressor, thus must be great enough to disrupt homeostasis, if the intent is to bring about an adaption [124]. Accordingly, METS can be divided into ranges of exercise intensities use for animals provided information about VO_{2max} is collected [1].

Table 11: METS and intensities for exercise experiments

Intensity	% of MET_{max}
Very Light	<30-35%
Light	30-50%
Moderate	45-65%
Hard	65-85%
Very Hard	≤85%

Table 11: METS and intensities for exercise experiments (adapted from[1]). MET_{max} indicates maximum METS calculated at VO_{2max}. $\%MET_{max}$ indicates the percentage of MET_{max} to work at.

Chapter 4: Future Directions and Reflections

I have been fortunate in that under Dr. Ouliana Ziouzenkova's guidance, I have been able to scientifically peruse combined interests of muscle metabolism, adipocyte biology, and exercise for the past two years. I arrived at Ohio State after numerous years in Division I football strength and conditioning to shift my focus on molecular and metabolic aspects of physiology and biology. Since joining her lab I have gotten the chance to begin dissecting research questions, which were once nothing more than thoughts in the back of my mind. She has begun to develop me into an independent researcher in areas such as study design, experimental implementation, and manuscript generation; and through these experiences I have grown to enjoy the process of making and learning from my mistakes more than the process of achieving initial experiment success.

This project arose though our phenotyping studies done on our alcohol dehydrogenase I (*Adh1*) mouse. When we discovered the mouse had large depots of epicardial adipose tissue accumulation, we then wanted to see if that had implications on its cardiovascular function. However, because the mice rely heavily on fat oxidation during most conditions, the PXT_m protocol was ineffective

at demonstrating they had impaired cardiovascular function. It was though this realization that I then went on to generate and optimize a new protocol that became the GXT_m.

The GXT_m was developed with the intention of being able to determine cardiovascular dysfunction during the phenotyping studies of uncharacterized models. It's ability to predict dysfunction was beyond my expectations as we saw with the *Adh1*$^{-/-}$ mouse, a model that previously had no known cardiac defects, had performance on the GXT_m that was similar to both dysfunctional models (**Fig.6**). Later studies done with the *Adh1*$^{-/-}$ using cardiac MRI went on to show that the mouse had significant differences in stroke volume and end diastolic volume compared to WT mice (unpublished data). Current studies are in progresses to further validate *Adh1*$^{-/-}$ mouse as a model with cardiovascular dysfunction that can be used to study epicardial fat accumulation.

Figure 6| Using the GXT_m to determine dysfunction in an unestablished model of cardiovascular dysfunction.

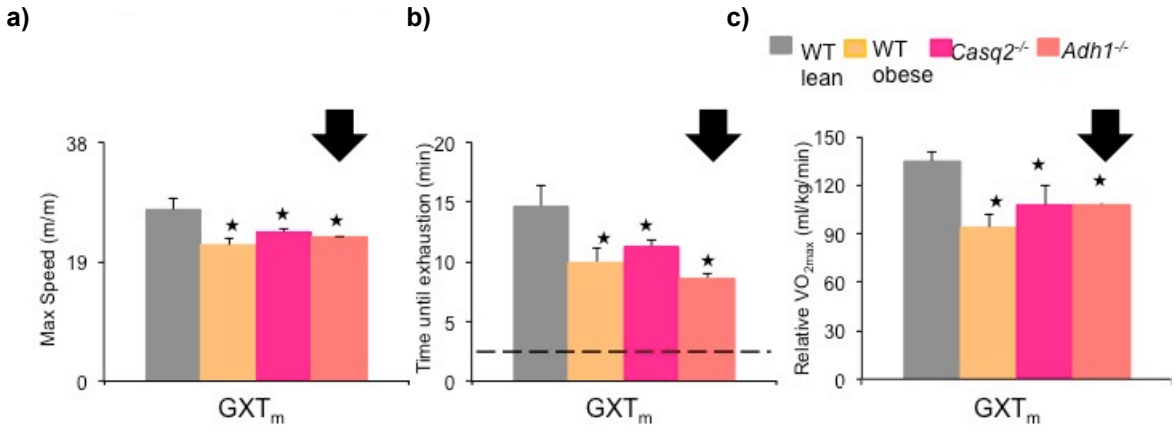


Figure 6| Using the GXT_m to determine dysfunction in an unestablished model of cardiovascular dysfunction. **(a)** When compared with dysfunctional models to WT animals, *Adh1*$^{-/-}$ also show decreased maximum speed **(b)** time until exhaustion **(c)** and relative $VO_{2max;}$ WT (grey), obese (orange), *Casq2*$^{-/-}$ mice (pink), and *Adh1*$^{-/-}$ (peach). Star indicates significance at the alpha = .007 level (MANOVA, multiple comparisons Tukey HSD for the PXT_m and GXT_m) and bar graphs represent mean ± SD; *n* = 26.

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