OMICS PUBLISHING GROUP/Clinical

Identification of Robust Cardiac Reference Genes in a mouse model of Cardiometabolic disease.

--Manuscript Draft--

Manuscript Number:	CLINICALGROUP-11-191R1
Full Title:	Identification of Robust Cardiac Reference Genes in a mouse model of Cardiometabolic disease.
Article Type:	Short Communication
Section/Category:	Journal of Clinical & Experimental Cardiology
Keywords:	Metabolic disease - Reference genes - High fat diets - Circadian Biology - YWHAZ - ACTB.
Corresponding Author:	Kimberley Bruce, Ph.D. University of Southampton Southampton, Hampshire UNITED KINGDOM
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	University of Southampton
Corresponding Author's Secondary Institution:	
First Author:	Kimberley Bruce, Ph.D.
First Author Secondary Information:	
All Authors:	Kimberley Bruce, Ph.D.
	Aaron J Stokes
	Nikesh R Patel
	Kerry Hyde
	Khaled H Sadek
	Mark A Hanson
	Christopher D Byrne
	Felino R Cagampang
All Authors Secondary Information:	
Manuscript Region of Origin:	UNITED KINGDOM
Suggested Reviewers:	
Opposed Reviewers:	
Response to Reviewers:	Response to reviewers JCEC I would like to thank both the reviewers for their comments and suggestions, which have been considered carefully. Response to reviewer one We are extremely grateful for your appreciation of the studies usefulness and careful analysis of the manuscript. 1)In response to the issue of reproducibility I have now included a statement concerning the intra-assay CV (coefficients of variability) values as less than 5 %. 2)Due to the nature of the Algorithm, the M values presented in the manuscript, which are a statistical product of the GeNorm programme, are a single value and therefore the median and error cannot be plotted. Since it is a marker of stability, I have now included an additional panel in Figure 1F which shows the mean Ct values for each

	housekeeping gene (when all the groups and time points are combined), to further illustrate that the stability is a measurement of less change, or data “spread” within each data set. This can be seen since the error bars become reduced as the M value becomes smaller and the RG is more stable. 3)If the reviewer refers to the “claim” as the fact that gene expression measurements may in inaccurate following unsuitable RG use, then we cannot give concrete examples since it would be wrong to highlight isolated authors work. We believe that this argument has its own conceptual merit currently appreciated and documented by many researchers in the field. 4)The Document has been checked for errors throughout and we thank the reviewer for their thorough analysis. Response to reviewer one We would like to thank reviewer 2 for their positive and useful comments. Whilst we wholly agree that the findings would be of wider scientific merit if additional models we included, we feel that this is beyond the scope of this particular manuscript. Our previous data (Bruce at al 2009) and soon to be published data (Torrens at el) shows that the high fat fed mouse in this model develop increased systolic blood pressure and endothelial dysfunction in addition to altered markers of inflammation and CVD. We also hope to address gene expression analysis with age in future studies. The manuscript has now been checked and the introduction revised.

Dear Editor

Journal of Clinical and experimental Cardiology

Please find attached our Article entitled "Identification of Robust Cardiac Reference Genes in a mouse model of Cardiometabolic disease", for consideration for short communication publication.

The incidence of cardiometabolic disease is rising rapidly, and will continue to rise along with the continuing obesity epidemic, and inappropriate lifestyles. There are few disease alleles or SNPs that confer a significantly increased risk of developing cardio metabolic disease components such as hypertension and CVD. Therefore, it is likely that diet induced stoichiometric alterations in gene expression in the heart ensue, and are capable of having a marked impact on long term organ function and disease pathogenesis. Although comparative gene expression studies are beginning to emerge in the literature, many do not include a suitable Reference gene (RG) selection strategy and therefore may include an internal control that also changes with experimental condition resulting in imprecise data. No studies have yet investigated the effect of cardiometabolic disease and time of day on RG expression. In this study we demonstrate that RG expression in the heart does indeed change in response to diet induced metabolic disease and time of day. In this study we show that the RGs; *YWHAZ* and *ACTB* (easily obtainable and may already be present in most laboratories) are constitutively expressed regardless of experimental condition. We recommend that in studies investigating the pathogenesis of CVD in a model of cardiometabolic disease and/or circadian disruption, that *YWHAZ* and *ACTB* (or an average of both) are used as internal controls to generate accurate data. Such investigations are important to understand the molecular pathogenesis of a growing and pertinent cardiometabolic disease state.

We would recommend this manuscript for short communication since it would provide researchers in this area a suitable RG indication, which is essential for generation of accurate data, and would conversely prevent the usage of unsuitable RGs, commonly used in the literature (*GAPDH*) at present.

Yours Sincerely

Kimberley Bruce

Identification of robust cardiac reference genes in a mouse model of cardiometabolic disease.

Kimberley D Bruce, Aaron J Stokes, Nikesh R Patel, Kerry Hyde, Khaled H Sadek, Mark A Hanson, Christopher D Byrne, Felino R Cagampang

Human Development and Health, Institute of Developmental Sciences, University of Southampton Faculty of Medicine, Southampton General Hospital (MP 887), Southampton SO16 6YD, UK.

Corresponding author:
Dr Kimberley Bruce
Human Development and Health
Institute of Developmental Sciences
University of Southampton
Faculty of Medicine
Southampton General Hospital (MP 887)
Southampton SO16 6YD
UK
tel: +44 2380798663
email: kdb@soton.ac.uk

Abbreviations used:

RG, reference genes; *GAPDH*, glyceraldehyde 3-phostphate dehydrogenase*; YWHAZ*, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein; *B2M*, beta-2 microglobulin; *EIF4A2*, eukaryotic translation initiation factor 4A2*; ATP5β*, ATP synthase H+ transporting mitochondrial F1 complex β subunit; *ACTB*, βeta actin; *CYC1*, Cytochrome C1; CVD, cardiovascular disease; MetS, metabolic syndrome; RT-PCR, reverse transcriptase polymerase chain reaction.

Abstract

Cardiovascular disease is linked to obesity, the metabolic syndrome, and altered 24hour (circadian) rhythms. Although the underlying mechanisms remain undefined, transcriptome analysis in the heart is beginning to provide important insights into the cardiometabolic pathogenesis. The reliability and accuracy of real-time quantitative PCR generated gene expression data is largely dependent on the selection of suitable reference genes (RG), which must be constitutively expressed regardless of cardio-metabolic disease state and time of day. However, many studies do not employ the appropriate selections strategies. In this study we determined the expression stability of seven candidate RGs (*GAPDH*, *YWHAZ*, *B2M, EIF4A2, ATP5β*, *ACTB* and *CYC1*) in a mouse model of diet-induced metabolic syndrome in both the day and night, using geNorm qBasePLUS software. RG expression varied in hearts of normal fed versus high fat fed mice, and was also dependant on the time of day. When all experimental variables were considered *YWHAZ* and *ACTB* were ranked the most stable and therefore the most suitable genes for generating comparative gene expression data in heart tissue from murine models of cardiometabolic disease. This study provides important information for reference gene selection, and will aid further transcriptome investigations into heart organ function.

Introduction

Transcriptome analysis of metabolic tissues can provide important insights into the pathogenesis of disease, aging, and the effectiveness of targeted interventions. Gene expression studies in the heart are particularly pertinent due to the current rise in cardiometabolic disease in an increasingly obese population. Obesity presents an increased risk of cardiovascular disease (CVD), in part through association with other features of the metabolic syndrome (MetS) such as insulin resistance, hypertension, dyslipidemia, fatty liver disease, pro-inflammatory state, and ectopic fat accumulation. Specifically type 2 diabetes, MetS and CVD coincide in 42.9-99% of individuals (1). Despite recognition of candidate disease gene loci (chromosome 3p and q), extremely few disease alleles (alpha-ketoglutarate-dependent dioxygenase FTO) have been identified (1;2). Therefore, it is likely that obesity induced alterations in gene expression stoichiometry within key metabolic tissues, such as the heart, play a major role in long term organ function. For example, preliminary evidence has shown that repetitive ischemia and reperfusion in diet induced obese mice is associated with significant down-regulation of fatty acid metabolism transcripts (3). Nonetheless the diet altered cardiac transcriptome is surprisingly uncharacterized. The lack of insightful gene expression studies may result from utilization of an inappropriate reference gene (RG) as an internal control.

Quantitative real-time PCR provides a sensitive, specific and reproducible means of determining comparative gene mRNA expression profiles. However, an increasing number of reports have shown that RT-PCR data accuracy is highly dependent on appropriate normalization strategies (4). It is now recognized that expression of classic RG can vary across tissue type and experimental condition (5). In addition, it has recently it has been shown that a significant number of metabolic genes in the heart are under the transcriptional control of an intrinsic circadian clock and 24hr expression pattern (6). Interestingly, specific disruption of the core molecular clock components can results in a MetS phenotype (7). Therefore, selection of a constitutively expressed cardiac RG, which maintains stability

irrespective of time of day or pathological conditions, is essential. In this study, we compared the expression the of seven RGs in the heart of control and high fat fed mice during both the night and day, in order to determine stably expressed RGs for use in gene expression studied investigating the pathogenesis of cardiometabolic disease.

Methods

Animal procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. C57/BL6J mice were used in this study. All animals were maintained under a 12h light/dark cycle (lights on at 07.00h), and at a constant temperature of 22±2°C. Newly-weaned offspring (3 weeks old) were randomly allocated to one of two diets: standard laboratory chow (control; 20.6% kcal fat; RM-1 Special Dietary Services, UK), or a high fat diet (45% kcal fat; Special Dietary Services, UK). At 15 weeks of age, offspring were killed by cervical dislocation at 2 time points during the 24h light-dark period at either 3 pm (Zeitgeber Time 8 or ZT8; eight hours into the light or day period), and at 3am (ZT20; eight hours into the dark or night period). Thus resulting in four groups: (1) control fed-daytime levels, (2) control fed-night time levels, (3) high fat fed-daytime levels, and (4) high fat fed-night time levels. Heart tissue was immediately snap frozen in liquid nitrogen and stored at -80°C.

Total RNA was isolated from 0.025g of whole heart tissue (n=5 per ZT and dietary group) using Trifast reagent (Peqlab, Germany) according to manufacturer's instructions. One microgram of total RNA from each sample was reverse transcribed to using M-MLV transcriptase (Promega, UK) was then added to each sample. Samples were then incubated at 37ºC for one hour, 42ºC for ten minutes, followed by an enzyme activation step at 75ºC for ten minutes.

Stability and expression of the RGs was determined at ZT8 and ZT20, otherwise referred to as day and night respectively, across both C and HF mice by quantitative real time reverse transcriptase polymerase chain reaction (qRT-PCR; Applied Biosystems 7500 Fast Real-Time PCR Thermal Cycler). The RGs, namely *ACTB*, *GAPDH*, *YWHAZ*, *B2M, EIF4A2, ATP5β* and *CYC1* (see Table 1) were taken from a geNorm Reference Gene Selection Kit with Perfect Probe (Primer Design, UK) and PCR reactions were prepared as according to instructions. PCR amplification was performed for 50 cycles. Following an initial enzyme activation step for 10 minutes at 95ºC, each cycle consisted of denaturation for 15 seconds at 95ºC, annealing for 30 seconds at 50ºC and 15 seconds at 72ºC. Analysis of duplicated samples indicate that the intra-assay coefficients of variability (CV) was >5%.

The stability in expression of RGs was analysed using qBasePLUS software (Biogazelle). We used qBasePLUS software version 1.5 based on geNorm and qBase technology. From this a stability measure (M) was generated by geometric averaging of multiple target genes and mean pair-wise variation of a gene from all other target genes in a given sample (8). Genes with the lowest M values are deemed the most stable.

Results

Methods used for RT-PCR are highly susceptible to error since small experimental variations have the capacity to produce large errors in gene quantification. For this reason, and to facilitate comparison of gene quantification between samples, it is important to normalise the expression of genes of interest against stable RGs; ideally highly expressed and unaffected by time, or experimental conditions. Such gene(s) can only be identified through an appropriate selection strategy specific for the tissue and set of experimental conditions (9).

We analysed the expression stability of each experimental group separately, in order to show the variation incurred due to high fat dietary intervention and time of day. Firstly, Figure 1A shows that *B2M* and *CYC1* are the two least stable, but *ATP5β* and *EIF4A2* are the most stably expressed housekeeping genes in heart tissue taken during the day from the control-fed group. Subtle alterations occur in heart tissues taken at night compared to tissues sampled during the day in the control-fed group and *YWHAZ* replaces *EIF4A2* as the most stably expressed gene (Figure 1B). High fat fed mice exhibiting features of the MetS, including significantly increased systolic blood pressure (10), show altered RG expression pattern during the day (Figure 1C). Notably, *ATP5β* reverts from high to lowest stability. Finally, heart tissue that was taken during the night from high fat fed mice (Figure 1D) also exhibit an altered RG expression pattern compared to tissues from control-fed animals (Figure 1B). Most notably, *CYC1* expression becomes relatively stable at night, whilst night time expression of *EIF4A2* destabilises. It is clear from this data that RG expression is subject to diet and circadian-induced alterations.

We also analysed the expression stability of all experimental groups in order to determine the most stable RGs overall (Figure 1E). Whilst *B2M* and *GAPDH* were the most unstable RGs, *YWHAZ* and *ACTB* were the most stable overall. In further support of this finding the mean Ct value for each RG is presented in Figure 1F, and clearly demonstrates that the error is reduced as stability of the gene increases (and M value decreases). For gene of interest in a cardiometabolic or circadian study, *B2M* would be a particularly unsuitable RG. In contrast, an average of the most stable genes (i.e. *YWHAZ* and *ACTB)* would be recommended to generate accurate comparative gene expression data.

Discussion

Few studies have investigated the appropriate selection of stable RGs in murine hearts under various experimental conditions, and none in a model of diet induced MetS and at different times of the day (5). Our study has demonstrated that murine heart RG expression

does indeed exhibit dietary and circadian modulations. In addition, we are the first to use an appropriate selection strategy to identify robust RGs, namely *ACTB* and *YWHAZ*.

These observed RG alterations are in concordance with recent findings. For example, *GAPDH* and *B2M* have been shown to vary according to the sex and age of the mice, and due to specific experimental conditions such as neurodegeneration and denervation status (11). We now show this variance is also observed according to diet and time of day, and a move away from classic RG which are influenced by these factors should be considered in designing experimental.

Until recently experiments involving heart failure have commonly used *GAPDH* and *18S ribosomal RNA* (*18S rRNA*) as reference genes (5). Previous work from our group has shown that RG expression within the hypothalamus of the brain are influenced by a HF diet intervention; hypothalamic *GAPDH* expression was significantly elevated in high fat-fed mice, whereas *ACTB* was found to be amongst the most stable (9). We also show that *GAPDH* vary in the heart in offspring challenged with a high fat diet at different times of the day. In confirmation, recent studies that have shown changes in *GAPDH* expression result from undernutrition and hyperinsulinemia (12). The protein product of *GAPDH* is a key glycolytic enzyme involved in carbohydrate metabolism (see Table 1). Therefore, *GAPDH* expression may be responsive to carbohydrate status thus explaining the instability of this RG in such studies involving nutritional interventions. Since sub-optimal nutrition and obesity are potential factors in the pathogenesis of heart failure it is likely that inaccurate data may be generated from inappropriate normalisation using RGs such as *B2M* and *GAPDH.*

Accurate gene of interest quantification is imperative to prevent erroneous experimental data. It is therefore surprising that 90% of gene analyse studies use only a single RG to normalise gene of interest data (13). RG are mostly chosen through inappropriate selection strategies, not taking experimental variation into account. Recent studies have highlighted

the role of altered circadian rhythms in cardiac physiology. Blood pressure, heart rate and cardiac output have all been shown to express a tightly controlled diurnal rhythm, as have a number of cardiovascular pathologies such as myocardial infarction (14). Epidemiological studies have shown that individuals with altered sleep wake cycles are at increased risk of developing MetS and CVD risk factors (15). Therefore, studies exploring the role of circadian physiology in CVD pathogenesis are timely, and at present the underlying molecular mechanisms are beginning to be investigated. We have shown that RG expression does indeed change across a light-dark period and must be considered in the experimental design.

In summary, we found *ACTB* and *YWHAZ* to be the most stable RG, not only in heart tissue of high fat fed mice exhibiting features of the human MetS but also across the 24h light-dark period. Therefore, for gene expression studies in metabolic or circadian models, an average of the most stable RGs (*YWHAZ* and *ACTB)* would be recommended for the most accurate comparative gene expression data. This study provides important information for reference gene selection, and will aid further transcriptome investigations into MetS, CVD and heart organ function.

Acknowledgments

This work is funded by the Biotechnology and Biological Sciences Research Council (BBSRC to FRC).

Disclosures: none.

Reference List

1. Bowden DW, Rudock M, Ziegler J et al. Coincident linkage of type 2 diabetes, metabolic syndrome, and measures of cardiovascular disease in a genome scan of the diabetes heart study. Diabetes 2006; 55(7):1985-1994.
2. Freathy RM, Timpson NJ, Lawlor DA et al. Common variation in the FTO gene alters diabetes-related metabolic traits to the extent expected given its effect on BMI. Diabetes 2008; 57(5):1419-1426.
3. Thakker GD, Frangogiannis NG, Zymek PT et al. Increased myocardial susceptibility to repetitive ischemia with high-fat diet-induced obesity. Obesity (Silver Spring) 2008; 16(12):2593-2600.
4. Martino A, Cabiati M, Campan M et al. Selection of reference genes for normalization of real-time PCR data in minipig heart failure model and evaluation of TNF-alpha mRNA expression. J Biotechnol 2011.
5. Brattelid T, Winer LH, Levy FO, Liestol K, Sejersted OM, Andersson KB. Reference gene alternatives to Gapdh in rodent and human heart failure gene expression studies. BMC Mol Biol 2010; 11:22.
6. Bray MS, Shaw CA, Moore MW et al. Disruption of the circadian clock within the cardiomyocyte influences myocardial contractile function, metabolism, and gene expression. Am J Physiol Heart Circ Physiol 2008; 294(2):H1036-H1047.
7. Froy O. The circadian clock and metabolism. Clin Sci (Lond) 2011; 120(2):65-72.
8. Vandesompele J, De PK, Pattyn F et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002; 3(7):research0034.1-0034.12.
9. Sellayah D, Sek K, Anthony FW, Hanson MA, Cagampang FR. Sensitivity of housekeeping genes in the hypothalamus to mismatch in diets between pre- and postnatal periods in mice. Neurosci Lett 2008; 447(1):54-57.

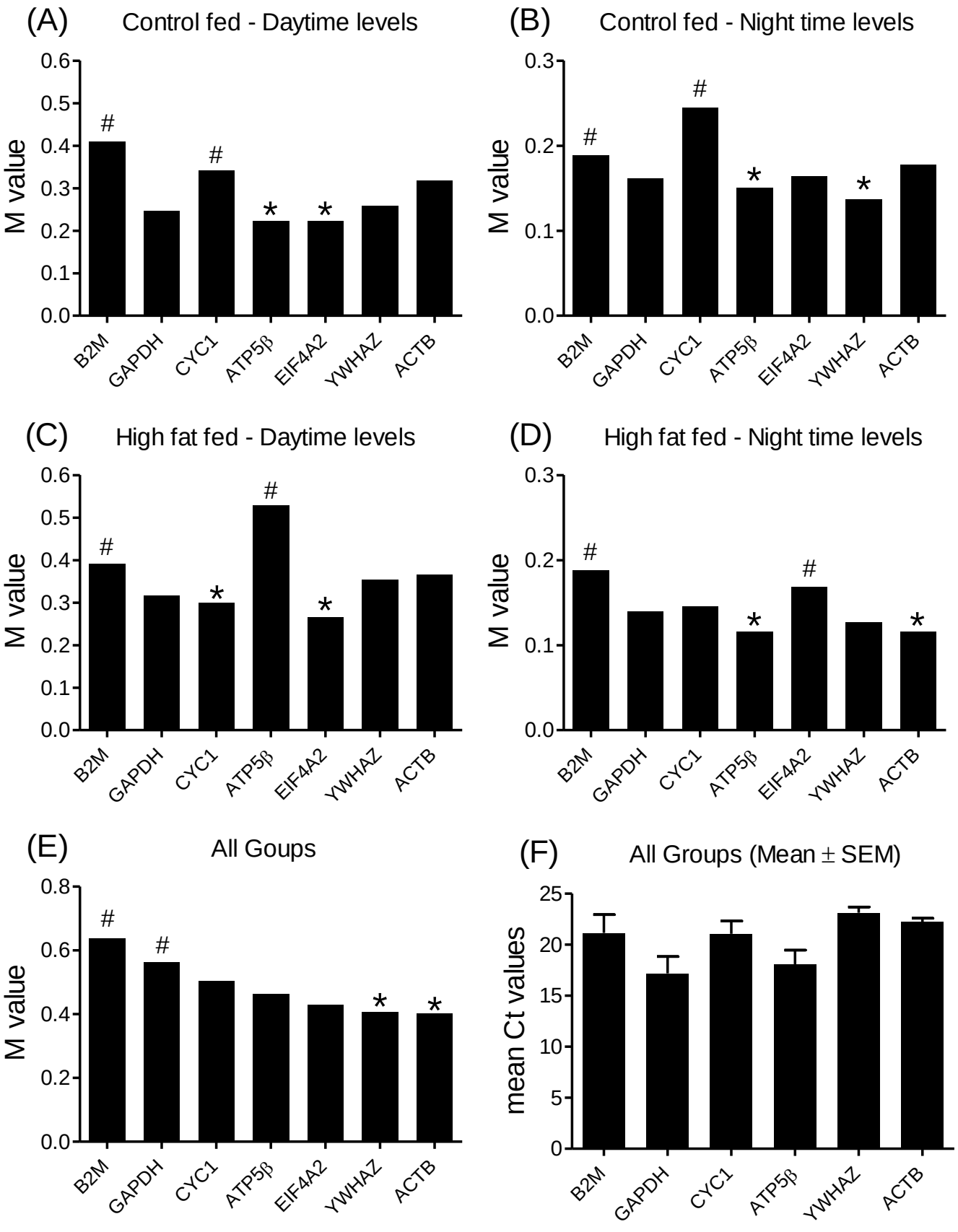
10. Bruce KD, Cagampang FR, Argenton M et al. Maternal high-fat feeding primes steatohepatitis in adult mice offspring, involving mitochondrial dysfunction and altered lipogenesis gene expression. Hepatology 2009; 50(6):1796-1808.
11. Manzano R, Toivonen JM, Calvo AC, Munoz MJ, Zaragoza P, Osta R. Housekeeping gene expression in myogenic cell cultures from neurodegeneration and denervation animal models. Biochem Biophys Res Commun 2011; 407(4):758-763.
12. Rhoads RP, McManaman C, Ingvartsen KL, Boisclair YR. The housekeeping genes GAPDH and cyclophilin are regulated by metabolic state in the liver of dairy cows. J Dairy Sci 2003; 86(11):3423-3429.
13. Suzuki T, Higgins PJ, Crawford DR. Control selection for RNA quantitation. Biotechniques 2000; 29(2):332-337.
14. Bray MS, Young ME. Diurnal variations in myocardial metabolism. Cardiovasc Res 2008; 79(2):228-237.
15. Davila EP, Florez H, Trepka MJ et al. Long work hours is associated with suboptimal glycemic control among US workers with diabetes. Am J Ind Med 2011; 54(5):375-383.

Table 1. Primary functions of RGs used.

Gene	Known function
ACTB	Ubiquitously expressed cytoskeletal protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase: converts D-glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate. Essential in glycolysis and carbohydrate metabolism.
YWHAZ	Binds to phosphoserine and phosphothreonine-containing proteins, mediating signal transduction.
B2M	Serum protein that associates with the major histocompatibility complex (MHC) class I heavy chain. Involved in antigen presentation to the immune system.
EIF4A2	Subunit of the eIF4F complex; ATP-dependent RNA helices required for mRNA binding to ribosomes.
ATP5beta	Subunit of mitochondrial membrane ATP synthase, which catalyses oxidative phosphorylation of ADP to ATP.
CYC1	Cytochrome c-1: heme-containing component of cytochrome b-c1 complex. Transfers electrons from Rieske protein to cytochrome c in the mitochondrial respiratory chain.

Figure legend

Figure 1. Stability of reference gene (RG) expression at either daytime or night time in the hearts of mice fed either a control (A and C) or high fat diet (B and D). Housekeeping gene expression in individual groups; * = the most stably expressed RGs, whereas # = the least stable RGs. (E.) Stability ranking for all groups. (F.) Mean Ct value±SEM for each gene when all groups and time points are combined.


(A)
Control fed - Daytime levels
M value
0.6
0.5
0.4
0.3
0.2
0.1
0.0
#
#
*
*
B2M
GAPDH
CYC1
ATP5β
EIF4A2
YWHAZ
ACTB
(B)
Control fed - Night time levels
M value
0.3
0.2
0.1
0.0
#
#
*
*
B2M
GAPDH
CYC1
ATP5β
EIF4A2
YWHAZ
ACTB
(C)
High fat fed - Daytime levels
M value
0.6
0.5
0.4
0.3
0.2
0.1
0.0
#
#
*
*
B2M
GAPDH
CYC1
ATP5β
EIF4A2
YWHAZ
ACTB
(D)
High fat fed - Night time levels
M value
0.3
0.2
0.1
0.0
#
#
*
*
B2M
GAPDH
CYC1
ATP5β
EIF4A2
YWHAZ
ACTB
(E)
All Goups
M value
0.8
0.6
0.4
0.2
0.0
#
#
*
*
B2M
GAPDH
CYC1
ATP5β
EIF4A2
YWHAZ
ACTB
(F)
All Groups (Mean ± SEM)
mean Ct values
25
20
15
10
5
0
B2M
GAPDH
CYC1
ATP5β
YWHAZ
ACTB