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TITLE:

Revealing Subtle Changes in Cardiac Function Using Transthoracic Dobutamine Stress Echocardiography in Mice

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KEYWORDS:

echocardiography, dobutamine, stress echocardiography, transthoracic echocardiography, mice, cardiac phenotyping, heart failure, animal cardiac imaging

SUMMARY:

Left ventricular dysfunction constitutes the final common pathway for a host of cardiac disorders. We here provide a detailed protocol of transthoracic dobutamine stress echocardiography approach for comprehensive evaluation of the left ventricular function of mouse models of cardiac disease as well as cardiac phenotyping.

ABSTRACT:

Left ventricular (LV) dysfunction paves the final pathway for a multitude of cardiac disorders. With the non-invasive high-frequency transthoracic dobutamine stress echocardiography in humans, a reductionist investigation approach to unmask subtle changes in cardiac function has become possible. Here, we provide a protocol for using this technique in mice to facilitate expanded analysis of LV architecture and function in physiology and pathology enabling the observation of alterations in models of cardiac disease hidden in unstressed hearts. This investigation can be performed in one and the same animal and allows both, basal and pharmacologically stress-induced measurements. We outline detailed criteria for appropriate anesthesia, imaging-based LV analysis, consideration of intra- and interobserver variability, and obtaining positive inotrope response that can be attained in mice after intraperitoneal injection of dobutamine under near physiological conditions. To recapitulate the characteristics of human physiology and disease in small animal models, we highlight critical pitfalls in evaluation, e.g., a pronounced Bowditch effect in mice. To further meet translational objectives, we compare stress-induced effects in humans and mice. When used in translational studies, attention must be paid to physiological differences between mice and human. Experimental rigor dictates that

some parameters assessed in patients can only be used with caution due to restrictions in spatial and temporal resolution in mouse models.

INTRODUCTION:

The hallmark of many cardiac diseases in human is a systolic and/or diastolic functional impairment of the left ventricle (LV). For the detection of structural abnormalities, the diagnosis, and the management of systolic heart failure as well as the evaluation of diastolic function in patients with symptoms of heart failure, echocardiography is used as a fundamental assessment modality.

Since the symptoms are unspecific and more than one third of patients with the clinical syndrome of heart failure may not suffer from the actual heart failure, it is important to find an objective echocardiographic correlate for the patient's clinical presentation[1]. Furthermore, some symptoms which are occult in the resting or static state may occur under conditions of activity or stress. In patients with coronary artery disease, already minor changes in coronary perfusion can lead to regional wall motion abnormalities. However, these subtle changes cannot be evaluated using conventional echocardiography as alterations of cardiac disease can be hidden in unstressed hearts. To gain a deeper understanding of the cardiac physiopathology, stress echocardiography provides a dynamic evaluation of myocardial structure and function under conditions of exercise or pharmacological induced stress, permitting matching symptoms with cardiac findings[2]. Also, in small animals, this method represents a non-invasive reliable in-vivo tool[3-5]. In-line with humans, stress reaction of the myocardium can be induced via pharmacological agents in mice and rats. Dobutamine is a frequently used drug and dobutamine stress echocardiography is widely performed in humans[6,7] but only sometimes used in small animal models to assess cardiac stress reaction[8-11]. Dobutamine is a synthetical catecholamine with a predominantly β1-agonistic effect resulting in positive inotropy and chronotropy of the heart. To achieve a correct translation from human to mouse, the technology and the conceptual framework of echocardiography, technical limitations related to e.g., the small size and rapid heart rate in the mouse must be considered. The human target heart rate in dobutamine stress echocardiography is [(220-age) x 0.85] resulting in an average heart rate increase of about 150 ± 10% in healthy volunteers[12,13]. For mice, such a formula is missing. The ejection fraction (EF) is described to be increased by stress echocardiography in humans by 5-20%[12,14]. The EF in mice is, depending on the heart rate, reported between 58 ± 11% (< 450 bpm) and 71 ± 11% (≥ 450 bpm) and changes by nearly 20% with higher heart rates[4]. The main mechanism in mice to increase the cardiac output is an increase in the heart rate. Partly responsible for this mechanism is the Bowditch effect or staircase phenomenon, a frequency-dependent calcium-mediated positive-inotropic cardiac response, that is more pronounced in mice than in humans[15,16]. In addition, (stress) echocardiography underlies intra- and interobserver variability. Therefore, a highly standardized procedure is indispensable[17,18].

Here we present the detailed procedure of dobutamine stress echocardiography to acquire standardized images to unravel subtle changes in cardiac function in mice in models of health and disease. Key components include adequate anesthesia, adequate heart rate monitoring and possible pitfalls in stress-induced imaging in mice. Key parameters are the evaluation of systolic

and diastolic function including consideration of the LVEF. Because mice are resistant to afterload-induced cardiac dysfunction[17], this protocol may add valuable information for the use in models of valvular heart disease as well.

PROTOCOL:

All methods and procedures were performed in accordance and compliance with all relevant regulations ('European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes' (Directive 2010/63/EU) and animal care was in accordance with institutional guidelines. Data from human subjects were analyzed in compliance with all institutional, national, and international guidelines for human welfare and was approved by the Local Ethics Committee (20-9218-BO). All experiments have been performed with male C57BL/6JRj at the age of 12 weeks.

1. Preparation of materials and equipment

NOTE: **Figure 1** shows an example of a small-animal ultrasound workplace.

1.1. Make sure to operate in a silent controlled environment with dimmable light.

1.2. Pre-heat the ultrasound gel, e.g., using a gel warmer. Allow the gel to warm to 37 °C. This may take a while.

1.3. Clean all instruments including the platform with a disinfectant wipe.

1.4. Turn on and pre-heat the platform to 37 °C.

1.5. Turn on the ultrasound machine. Enter the animal ID and protocol ID as well as other relevant information. Use a high-frequency ultrasound transducer with a center transmit of 30 MHz for mice with approximately 30 g body weight.

1.6. Make sure to work with an active gas exhaustion system.

NOTE: If using an activated charcoal filter for adsorbing the isoflurane in the exhaled flow, make sure to check the weight and replace the filter once the indicated maximal weight increase is reached.

1.7. If necessary, fill the vaporizer with the adequate amount of isoflurane.

CAUTION: Do not inhale volatile anesthetics.

1.8. Prepare a dobutamine working solution of 2.5 µg/µL either by dilution of a ready-to-use injection solution or by dissolving dobutamine hydrochloride powder in 0.9% saline according to the manufacturer's instruction. The solution is stable at least for 24 h when stored at room temperature.

[Place **Figure 1** here]

2. Preparation of the mouse for imaging and induction of anesthesia

2.1. Fill the induction chamber with 3%-4% v/v isoflurane in an oxygen-enriched gas mixture (with 1 L/min 100% O_2).

2.2. Weigh the mouse. Gently pick up the mouse by the tail and transfer it to the induction chamber. Ensure that the animal is sedated within a few seconds by closely observing the animal's movements.

2.3. If necessary, change the gas flow to the nose cone connected to the anesthesia system (1.0-1.5 vol% isoflurane with 1 L/min 100 % O_2 to maintain a stable sedation). Remove the mouse from the induction chamber and place it carefully on the pre-heated platform. Ensure that the paws lie on the ECG sensors embedded in the platform.

2.4. To prevent drying of the sclera, apply ointment gel to both eyes.

NOTE: Stress measurements will take their time.

2.5. Apply a very small amount of electrode cream to the ECG sensors. Gently secure the animal with adhesive tape on all four limbs. Use a small part of adhesive tape to secure the animal's head position in the nose cone. The ECG is used to record the heart rate during image acquisition. Adjust the physiological imaging system for a stable and clear ECG signal.

NOTE: Too much electrode cream may result in bad ECG signal quality.

2.6. To protect the animal from stress during the procedure, check the adequate depth of sedation by maintaining the heart rate range at 400-450 bpm. The heart rate is obtained by the ECG. A variation of 50 bpm within the range is acceptable.

NOTE: Movements of the animal may indicate a too narrow level of sedation. The anesthesia must not lead to cardio-depression of the mouse. The sedation can be adjusted to obtain the above-mentioned target heart rate.

2.7. Using lubrication, gently insert a rectal thermometer for continuous monitoring of the body temperature. Keep the temperature in the physiological range (normally between 36.5 °C and 37.5 °C depending on the mouse strain and experimental setup). In a non-environmentally controlled animal cardiac ultrasound laboratory, the use of infrared lighting may be considered.

2.8. Use chemical depilatory cream to remove the body hair from the chest. Use a clean damp paper towel to wipe clean the chest. Make sure to remove all remaining cream components (**Figure 2A**).

NOTE: An electric clipper can be used before depilatory application as well. The animal is now prepared for imaging. As it is critical to keep imaging time short, the whole preparation before imaging should take less than 3 min.

[Place **Figure 2** here]

3. Basic cardiovascular imaging

NOTE: Images can be acquired using two basic transducer positions (parasternal and apical ultrasound window) (**Figure 2**) and at least three ultrasound modalities (B(rightness)-mode, M(otion)-mode and Doppler-mode (color doppler and pulsed-wave (PW) doppler) (**Figures 3-5**). For basics of imaging please refer to previously published articles[16,18]. It is critical to obtain clear images for the comparison with later acquired stress images.

3.1. Apply pre-heated ultrasound gel bubble-free to the chest.

NOTE: Non-heated ultrasound gel will result in rapid body temperature loss which will affect the heart rate.

3.2. Perform parasternal long axis (PSLAX) view.

NOTE: PSLAX is performed to visualize the LV in its long axis. With this, e.g., the aortic root dimensions and proximal aorta dimension as well as the LV length can be obtained.

3.2.1. With the head facing away from the investigator, tilt the table approximately 10-20° to the left and 5-10° to the front to bring the heart as anterior as possible. Place the transducer parasternally in line with the long axis of the heart with the marker (notch) pointing towards the animal's right shoulder (**Figure 2B**).

3.2.2. Use micromanipulators to adjust the optimal view. Use image control panel controls to optimize the image. Acquire at least one 2D B-mode picture and one M-mode picture in midventricular level.

3.2.3. Acquire any additional images if need for the specific question. Acquire at least 100 frames and at least 3(-6) full cardiac cycles.

3.3. Perform parasternal short axis (PSSAX) view.

NOTE: PSSAX is performed to visualize the LV in its short axis. From this view, e.g., left-ventricular end-systolic volume (LVESV), left-ventricular end-diastolic volume (LVEDV), stroke volume (SV) and cardiac output (CO) can be calculated.

3.3.1. Rotate the transducer 90° clockwise without changing of the angulation (marker now

pointing towards the animal's left shoulder) (**Figure 2C**). Acquire at least one B-mode image in the basal, midventricular (level of the papillary muscles), and apical view.

3.3.2. To define the most basal and the most apical view, scroll along the long axis to the most distant points, where the full cardiac cycle of the LV chamber is still visible. Take images on the midventricular level approximately in an intermediate position at the level of the papillary muscles.

3.3.3. Acquire at least one M-mode image in the midventricular view.

NOTE: Some ultrasound machines provide presets for the different views; it is recommended to check for the adequate preset before acquiring images.

3.4. Perform apical four chamber (4CH) view.

NOTE: 4CH is important because it can be primarily used to evaluate the mitral valve using PW doppler.

3.4.1. Tilt the platform that the animal is in a modified Trendelenburg position with the head down. Angle the transducer towards the head of the mouse, the marker facing towards the animal's left side (**Figure 2D**).

3.4.2. Acquire at least one B-mode image as well as color doppler and PW doppler image of the mitral- and tricuspid valve. Depending on the experimental question, apply tissue doppler in the 4CH view.

NOTE: The easiest way to reach apical 4CH position is to tilt the table from the PSAX view and to angulate the transducer. Be careful not to apply too much pressure to the thorax as this may interfere with measurements e.g. of diastolic function.

4. Dobutamine stress imaging

NOTE: Once the target heart rate is reached, standardized views should be acquired as long as the target heart rate is stable. This typically requires more than one switch between PLAX and PSAX. Because the switch between PSLAX and PSSAX only requires a 90° rotation, the views can be imaged easily.

4.1. Perform Dobutamine stress imaging in one and the same animal maintaining the same anesthesia to ensure comparability. Make sure the starting heart rate remains stable in the range of 400-450 bpm. Record the ECG readings and save it together with and on the acquired images. Make sure the ECG signal is clear. Otherwise, try to re-tape all four limbs until a clear ECG signal is displayed.

4.2. Again, perform PSLAX view (B-mode and M-mode images). Save the images as "baseline"

image. Be sure, to save and keep in mind the initial heart rate as well.

4.3. Prefill the syringe and inject 5 µg/g body weight dobutamine intraperitoneally using a 27 G needle and 1 mL syringe. Closely watch for the heart rate. Record echocardiographic images until the target heart rate is reached and use the increase of the heart rate for later analysis. A sustainable significant dobutamine-induced heart rate increase is reached after an increase of 15-30% after about 1 min, depending on the dobutamine dose.

NOTE: Always use single-use sterile injection needles for every animal to prevent from infections. The dobutamine susceptibility and the (sub)maximal load may vary with the mouse strain and may be dependent on the experimental setup and should be defined in pre-experiments. It is recommended adjusting the dobutamine dose to the experimental setup.

CAUTION: Follow institutional guidelines for the use of sharp and potential infectious items. Always dispose the needle into an approved medical waste container!

4.4. Once the target heart rate is reached and remains stable for approximately 30 s, acquire PSLAX B-mode and M-mode images as described in step 3.

4.5. Again, rotate the transducer clockwise to obtain PSSAX view as described in step 3. Here, acquire B-mode images of the basal, midventricular (level of papillary muscles) and apical level and M-mode images of the midventricular (level of the papillary muscles) level. Reassure, that the target heart rate remains stable. Otherwise, switch back to PSLAX position and start imaging again.

NOTE: As the heart rate will drop without continuous infusion of dobutamine[12] (not covered in this article), the images should be acquired within two minutes. PLAX and PSAX images are essential for most of the relevant stress-induced measurements (see “Representative Results” section).

4.6. Now, perform apical 4CH view again (as explained in step 3.4.). Using PW doppler, measure the flow patterns of interest (as explained in step 3.4.2.). Under unstressed conditions, two characteristic waves are measured using PW doppler, one representing the passive filling of the ventricle (E(arly) wave) and one representing the active filling after atrial contraction (A(trial) wave). With increasing heart rates, these waves tend to fuse and may not be measurable distinctively under dobutamine induced stress.

5. Final steps

5.1. After approximately 5 min when the heart rate will start to decrease again, make sure all views are captured.

5.2. Gently remove the ultrasound gel from the chest using a clean damp paper towel. Carefully remove the tape fixation. Pay special attention to the tape fixing the animal’s head to avoid

pulling out the whiskers.

5.3. Turn off the anesthesia. If using an active gas exhaust, make sure to continue gas exhaustion. Place the animal on a paper towel in a separated heated cage during the wake-up period. Observe the animal closely. It must not be left unattended until it has regained sufficient consciousness to maintain sternal recumbency. Once awake and fully recovered, transfer the animal to its cage.

NOTE: Due to the non-final character of this technique, the animal may stay within the experiment in accordance with all relevant regulations.

6. Offline evaluation

6.1. Transfer the image data to the offline analysis software on a working-station to perform detailed evaluation of the cardiac function. Pay special attention to the difference between unstressed and stressed heart function. The heart rate should always be recorded and presented.

NOTE: Because software analysis varies between different software, it is not covered in this protocol. Please refer to the manufacturer's instructions.

REPRESENTATIVE RESULTS:

A physiological unstressed echocardiographic image acquired in PSLAX is shown in **Figure 3**. In diastole, the ventricle walls appear uniformly (**Figure 3A**) and thicken to a certain degree (**Figure 3B,C).** The injection of 5 µg/g body weight dobutamine i.p. leads to a significant increase of the heart rate (positive chronotropic effect)[12] and the LVEF (positive inotropic effect) (**Figure 3, Figure 6**). The positive inotropic effect is visualized in **Figure 3D-F** with a thickening of the LV anterior wall in diastole (LVAWd) and even more pronounced in systole (LVAWs) and the LV posterior wall in diastole and systole (LVPWd; LVPWs) (**Figure 3D,E**). The effect could lead to a "kissing walls" phenomenon (**Figure 3F**) where the LV inner diameter (LVIDs) shortens in such an extent that the anterior and posterior walls seem to touch themselves (**Figure 3E**). The LVEF can be measured planimetrically in PSLAX B-mode (**Figure 3A,B,D,E)**.

In PSSAX, the septal wall (LVSW) and lateral wall (LVLW) are visualized in addition to the LVAW and the LVPW (**Figure 4**). In the stressed heart, the ventricle contracts circumferential uniformly towards its center (**Figure 4D,E**). The "kissing walls" phenomenon can be seen in PSSAX M-mode as well (**Figure 4F**).

The PW doppler recording of the mitral valve flow profile shows the early diastolic velocity (E wave) and the late diastolic atrial contraction (A wave) with a distinct separation of E and A wave (**Figure 5A**). The isovolumetric relaxation time (IVRT) is needed for evaluation of the diastolic function and the isovolumetric contraction time (IVCT) represents the systolic function. In a healthy stressed heart, both parameters decrease significantly (**Figure 6C,D**). **Figure 5B** shows nearly fused E and A waves which can be seen with increasing heart rates. Please note that due to the significant decrease of the LVID in systole, outflow tract signals can be seen when measuring the mitral valve flow (**Figure 5B**).

Using specialized software, the speckle-tracking assessment of the strain and strain rate of the LV myocardium is possible in unstressed and stressed hearts (**Figure 7, Figure 8**) to measure early or sub-clinical chances in intrinsic myocardial contractile properties. It is important to perform calculations with the precisely recognized parts of the contraction cycle. Strain reflects the deformation of an object normalized to its original shape and size[4] which equals the length of the myocardial fiber normalized to its original length[6], strain rate represents the myocardial deformation rate. Strain can be measured in the radial, circumferential and longitudinal axes. One example is the measurement of the radial strain in PSSAX, which describes the increase of the myocardial wall thickness during the cardiac cycle and represents its deformation towards the center of the left ventricle[19]. The cardiac systolic synchrony is a measure of the systolic function of six LV segments (**Figure 9E**)[20,21]. In humans, the clinically used global longitudinal strain (GLS) is an accepted overall marker for evaluation of the systolic function.

In stress echocardiography in mice, we observed an increase in GLS (measured in PSLAX) to less negative values and a decrease in global circumferential strain (GCS) (measured in PSSAX) to more negative values (**Figure 9A,B**). Dobutamine administration reduced the global time to peak strain (**Figure 9C**) which is represented in a reduced time to peak strain across all six segments (**Figure 9D**). It furthermore led to a uniformly increase in radial strain rate over all segments (**Figure 9F**).

The results of dobutamine administration vary between species, which is important for translational approaches. Once, (sub)maximal load is reached, mice showed an average heart rate increase of +33% while humans showed an average heart rate increase of +144 % (**Figure 10A**). At (sub)maximal load, mice showed an average dobutamine-induced EF increase of +61 %, humans showed an average EF increase of +14% (**Figure 10B**).

FIGURE AND TABLE LEGENDS:

Figure 1: Small-animal cardiac ultrasound workplace. An ergonomic setting is indispensable for small-animal stress echocardiography as examination times must remain short. The workplace consists of an ultrasound machine, a small-animal anesthesia system with oxygen supply and active gas exhaust, a heated echocardiography platform with embedded ECG and movement capabilities *via* micromanipulators as part of an integrated rail system as well as a physiological monitoring unit. A gel warmer to use ultrasound gel and a heat lamp are useful aids.

Figure 2: Animal and transducer positioning. (**A**) The mouse is attached to the heated platform with all four limbs fixed on the silver ECG electrodes. A rectal thermometer is inserted for body temperature measurement. The snout is gently inserted to the nose cone of the anesthesia system. (**B**) Probe orientation for parasternal long axis view (PSLAX); see step 3.2. (**C**) Probe orientation for parasternal short axis view (PSSAX); see step 3.3. (**D**) Probe orientation for apical four chamber view (4CH); see step 3.4.

Figure 3: Representative results of images acquired in parasternal long axis (PSLAX) in the

unstressed and stressed animal. (**A**) PSLAX in diastole (unstressed). Visible are the left ventricular anterior wall (LVAWd), left ventricular posterior wall (LVPWd), the LV cavity and the aortic valve (Ao). (**B**) PSLAX in systole (unstressed). Visible are the left ventricular anterior wall (LVAWs), left ventricular posterior wall (LVPWs), the LV cavity and the Ao. (**C**) M-mode image (unstressed) of midventricular PSLAX. Visible are LVAWd; inner ventricular diameter in diastole (LVIVDd), LVPWd, LVAWs, LVIVDs, LVPWs. Heart rate of unstressed images: 425 bpm. (**D**) PSLAX in diastole (stressed). Visible are the LVAWd, LVPWd, the LV cavity and the Ao. (**E**) PSLAX in systole (stressed). Visible are LVAWs, LVPWs, the LV cavity and the Ao. (**F**) M-mode image (stressed) of midventricular PSLAX. Visible are LVAWd, LVIVDd, LVPWd, LVAWs, LVIVDs, LVPWs. Heart rate of stressed images: 545 bpm.

Figure 4: Representative results of images acquired in parasternal short axis (PSSAX) in the unstressed and stressed animal. (**A**) PSSAX in diastole (unstressed). Visible are the left ventricular anterior wall (LVAWd), left ventricular posterior wall (LVPWd), the left ventricular inner diameter (LVIDd; arrows), the left ventricular septal wall (LVSWd) and the left ventricular later wall (LVLWd). (**B**) PSSAX in systole (unstressed). Visible are the left ventricular anterior wall (LVAWs), left ventricular posterior wall (LVPWs), the left ventricular inner diameter (LVIDs; arrows), the left ventricular septal wall (LVSWs) and the left ventricular lateral wall (LVLWs). (**C**) M-mode image (unstressed) of midventricular PSSAX. Visible are LVAWd, LVIVDd (arrows), LVPWd, LVAWs, LVIVDs (arrows), LVPWs. Heart rate of unstressed images: 400 bpm. (**D**) PSSAX in diastole (stressed). Visible are the LVAWd, LVPWd, LVIDd (arrows), LVSWd and the LVLWd. (**E**) PSSAX in systole (stressed). Visible are the LVAWs, LVPWs, LVIDs (arrows), LVSWs and the LVLWs. (**F**) M-mode image (stressed) of midventricular PSLAX. Visible are LVAWd, LVIVDs, LVPWd, LVAWs, LVIVDs, LVPWs. Heart rate of stressed images: 550 bpm.

Figure 5: Representative results of images acquired in apical four chamber view (4CH) in the unstressed and stressed animal: MV flow. (**A**) Pulsed wave doppler of mitral valve inflow (unstressed). The E/A ratio can be derived from the E(arly) diastolic ventricle filling and the A(trial) diastolic contraction. Isovolumetric relaxation time (IVRT) serves as a marker for the diastolic function whereas isovolumetric contraction time (IVCT) represents the systole. To visualize IVRT and IVCT adequately, lower the wall filter of the ultrasound machine when acquiring the images. Heart rate of the unstressed image: 400 bpm. (**B**) Pulsed wave doppler of mitral valve inflow (stressed). With increasing heart rates, E and A wave approximate in high and furthermore tend to fuse. When acquiring dobutamine-induced stress echocardiography, outflow signals (*) may become visible due to the stress-induced decrease in left ventricular cavity size. Heart rate of stressed image: 560 bpm.

Figure 6: Changes in cardiac functional parameters in dobutamine-induced stress echocardiography. (**A**) Increase of the heart rate in beats per minute (bpm). (**B**) Increase of left ventricular ejection fraction. (**C**) Shortening of isovolumetric relaxation time (IVRT). (**D**) Shortening of isovolumetric contraction time (IVCT). All: n = 3, mean ± SD, * = $p < 0.05$, student's t-test.

Figure 7: Strain analysis of images acquired in parasternal long axis view (PSLAX) in unstressed

and stressed animals. (**A,B**) Using strain analysis software, calculation of cardiac functional parameters including global longitudinal strain (GLS) is possible. In the healthy animal, the wall signals are uniform (red/blue pattern).

Figure 8: Strain analysis of images acquired in parasternal short axis view (PSSAX) in unstressed and stressed animals. (**A,B**) Using strain analysis software, calculation of cardiac functional parameters including global circumferential strain (GCS) is possible. In the healthy animal, the wall signals are uniform (red/blue pattern).

Figure 9: Changes in strain parameters in unstressed and stressed animals. (**A**) Decrease of global longitudinal strain. (**B**) Increase of global circumferential strain. (**C**) Reduced global time to peak strain. (**D**) Reduced time to peak strain across all six segments. (**E**) Schematic overview of anatomical segments in short axis view. (**F**) Increased radial strain rate after dobutamine administration. As values are software-dependent and referent values in mice are currently not present, the values must be seen individually for each experimental setup. The graphs represent the result of Figure 7 and 8. AFW = anterior free wall; LW = lateral wall; PW = posterior wall; IFW = inferior free wall; PS = posterior septum; AS = anterior septum.

Figure 10: Differences between mouse and human cardiac functional parameters in dobutamine-induced stress echocardiography. (**A**) percentual increase of heart rate. (**B**) percentual increase of left ventricular ejection fraction. The values derive from literature[13,22] as well as clinical routine measurements. All: n = 3, mean ± SD.

DISCUSSION:

Stress-induced evaluation of the cardiac function is widely used in humans in a clinical setting using exercise testing or pharmacological stress testing[6,7]. Because immediate post-exercise echocardiography of mice is very limited due to the need for sedation, dobutamine-induced stress echocardiography is likely to be the most translational method to assess stress-induced cardiac physiopathology. Reliable information on cardiac function can be obtained using real-time pressure-volume analysis in a final approach[23]. Echocardiography adds a valuable insight into long-term examination and, therefore, additionally meets the requirements of the 3R principle (replace, reduce, refine).

The most common use of stress echocardiography in patients is the search for wall abnormalities and myocardial viability as well as the evaluation of valvular heart disease and their importance is indicated in recent guidelines[24,25]. Nevertheless, those applications are only a piece of the physiopathological puzzle of a failing heart. We, therefore, believe that the use of pharmacological stress testing in preclinical research will help to uncover hidden and subtle changes in the cardiac function in models of disease and in cardiac phenotyping.

Anesthesia and heart rate

With a few exceptions of conscious echocardiography in mice[26], sedated echocardiography is the most widespread echocardiographic method in mice[3,4]. Although up to 1.5 vol% isoflurane will be enough for a proper sedation, isoflurane – in contrast to humans – leads to a decrease of heart

rate in mice. Therefore, it is crucial to find the right balance between sedation and heart rate influence, especially when performing stress testing. Nevertheless, isoflurane causes less cardiodepression compared to other anesthetics[27]. The physiological heart rate of mice is >450 bpm[4] and with respect to a slightly reduced heart rate due to anesthesia, we defined a target heart rate of 400-450 bpm as a suitable target heart rate of unstressed mice to obtain a significant increase in heart rate after dobutamine injection. Therefore, careful monitoring of the heart rate is indispensable as it serves as primary control mechanism of adequate anesthesia and as verification of a successful dobutamine effect when reaching the pre-defined heart rate increase after dobutamine injection. At this point, it is important to use the same heart rate corridor within one group of mice and compared to another group of mice. One should note that the effect of dobutamine is more inotropic than chronotropic.

Dobutamine administration

In mice echocardiography, dobutamine is administered i.v. or i.p. with the dose ranging from 1-5 µg/g body weight[11,28-30]. Dosing is critical, as dobutamine plasma concentrations and cardiac functional parameters correlate in a linear relationship[13]. We here decided for i.p. injection of 5 µg/g body weight because it showed a significant, robust, reproducible and transient increase in cardiac functional parameters reaching the (sub)maximal load in male C57BL6/6JRj mice used. Because the protocol is, unlike to human patients, not searching for interindividual differences within the experimental mice group, the same (sub)maximal load should be reached in every mouse examined requiring the same application dose in every animal. At this point we would like to point out that dose-response testing until a significant, robust, reproducible and transient increase in cardiac functional parameters is reached should be done dependent on the mice strain and the experimental setting beforehand.

I.p. injection is a feasible administrative route as i.v. injection requires higher effort in mice. Furthermore, assuming the comparable peritoneal resorption rate of dobutamine in mice, the i.p. injection approach avoids technical barriers and inaccuracies in i.v. tail vein injection, especially when serial testing is performed in longitudinal experiments, which would require several tail vein injections of previously punctured vessels. Nevertheless, similar to human procedures, an i.v. application approach could also be adapted[9,27,31]. Because of different pharmacodynamics and pharmacokinetics, this may require an additional dose-response testing in advance. As predominantly selective β_1-adrenergic drug, dobutamine is often chosen due to its cardiac inotropic and chronotropic effects with minimal direct effect on the blood pressure in preclinical studies[9]. As an alternative to dobutamine, the administration of isoproterenol is possible. Isoproterenol is a non-selective β-adrenergic agonist with effects on both β_1- and β_2-adrenergic receptors, which – in contrast to dobutamine – causes a lowering of the mean arterial pressure after application due to vasodilatation. It leads to cardiac hypertrophy and fibrosis and is used to mimic stress-induced cardiac injury in a rather acute and as heart failure model in a rather chronic administration[9,32], so that it is somewhat inferior to dobutamine when repeated stress echocardiography is planned in a longitudinal animal experiment. Nevertheless, the comparison of both substances did not show significant differences in inotropic and lusitropic effects in healthy mice[33]. Overall, dobutamine is more commonly used for stress testing in the clinical setting, while isoproterenol is seen in the experimental setting[27,33].

Evaluation of cardiac function

Stress-induced changes can be assessed visually in B-mode and with benefits in spatiotemporal solution in M-mode, making it somewhat superior in stress imaging. In untreated healthy mice or mice without wall abnormalities like models of chemotherapeutic-induced cardiotoxicity[19], M-mode images of the midventricular level acquired in PSLAX or PSSAX can be used to adequately measure LV systolic function[34]. However, in the presence of regional wall (motion) abnormalities, e.g. after myocardial infarction[35], the planimetric measurement in B-mode images may be preferable to assess myocardial contractile reserve.

Despite excessive research, diastolic heart failure with preserved EF still is an unsolved entity of heart failure[36]. For deeper understanding of underlying mechanisms, the analysis of small animal models of diastolic heart failure is indespensable[37]. For diastolic evaluation, markers like mitral valve inflow patterns, IVRT and IVCT are well established markers[4,38]. In stress testing, those markers are subjected to change. Due to missing standard values in stress testing in mice, the changes must be closely compared to the non-treated control group. Our findings may serve as orienting values. While we already have increased the sensitivity to detect subtle and early changes in myocardial function by stress testing, the sensitivity can be further increased using speckle-tracking imaging as myocardial strain may detect early sub-clinical changes beforehand[39]. As shown in our representative results, strain-derived markers may change by stress testing, which is of interest when comparing models of cardiac diseases like myocardial infarction, where segmental analyses become important. With the current absence of reference values despite efforts for standardization of echocardiography in mice[4], these effects should be reassessed for every individual experimental setting. Attention in stress testing strain analysis is needed, as the analysis of high heart rates and significantly induced cardiac function parameters depends on calculations performed with the accurately recognized parts of the contraction cycle.

Intra- and interobserver reliability

As an operator-dependent ultrasound examination, echocardiography underlies possible intra- and interobserver variability[3], especially when performed in one and the same animal. A well-respected guide to assess the reproducibility of echocardiographic measurements has been established[40]. With emerging software techniques, automated assessment algorithms have been developed[41]. We here recommend a blinded image acquisition as well as blinded software-based offline image interpretation, whenever possible. To assure reproducibility especially in stress echocardiography, images with a comparable heart rate increase should be analyzed.

Between mice and human: Translational application

With all advantages and evolution of preclinical ultrasound application, there remains the question of translational limitations.

As we have shown, the effects of dobutamine vary between mice and humans. Translational preclinical researchers should not expect the same increase of cardiac functional parameters they are used to in human patients or vice versa. If, however, a comparison should be performed, we suggest only to compare the points of (sub)maximal load. A more pronounced Bowditch

effect[15,16] has to be kept in mind when translating preclinical echocardiographic measurements.

Furthermore, due to limitations because of small ultrasound windows, the changes after dobutamine injection in heart rate and contractility, evaluations of e.g. stress assessment of the pulmonary circulation[42], cannot be performed in mice without further ado. Further research is needed to assess strain evaluation in mice. Nevertheless, dobutamine-induced stress-echocardiography is a feasible, reproduceable and valuable tool for unmasking hidden subtle changes in the preclinical research area.

The application of our protocol allows for biologically highly relevant insights into systolic and diastolic dysfunction of the heart and can besides cardiac phenotyping of different mouse strains be expanded to distinct models of chronic overload pressure, myocardial infarction, diastolic dysfunction and heart failure with preserved LVEF as well as valvular heart disease.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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Figure 2

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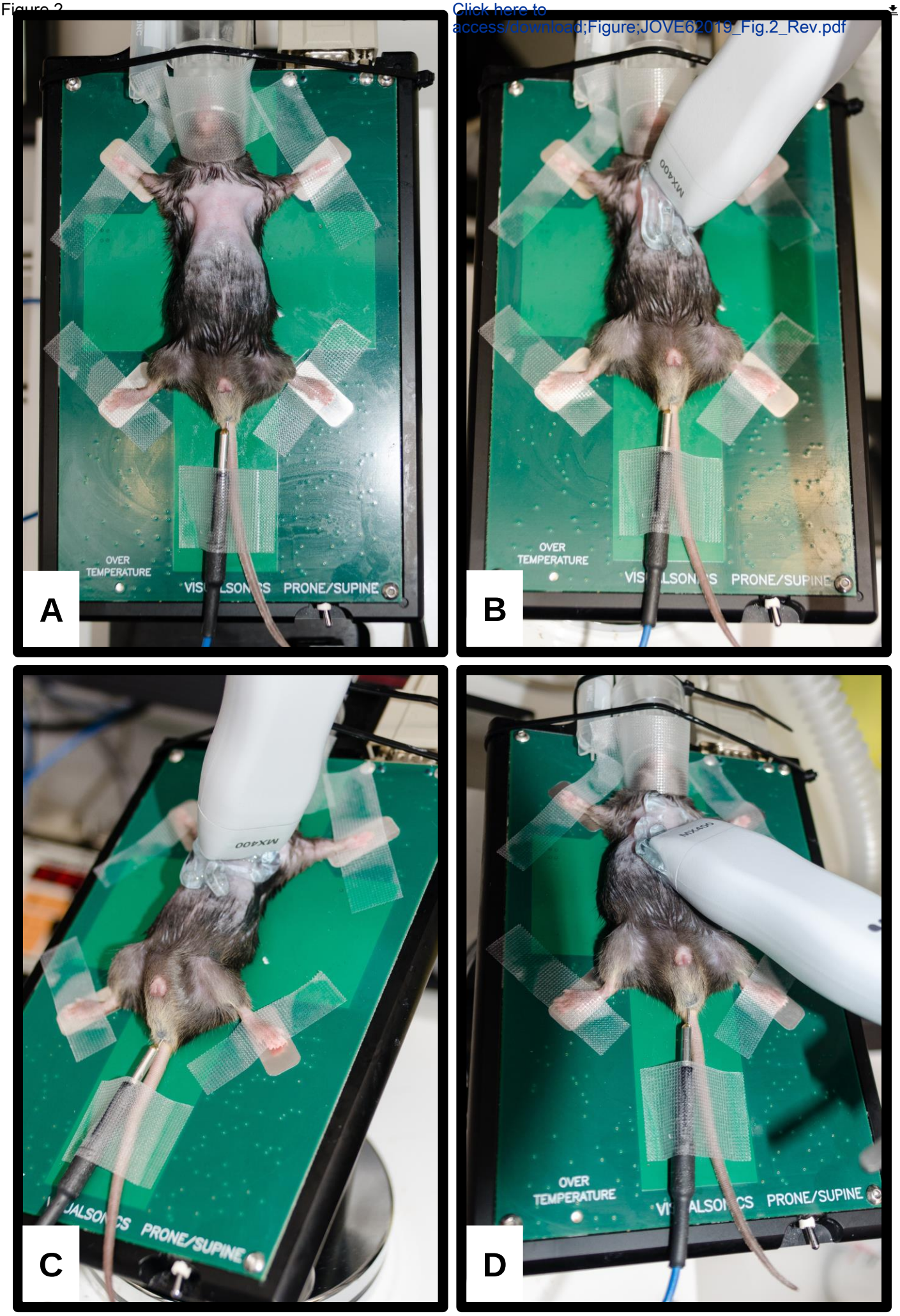

Figure 3

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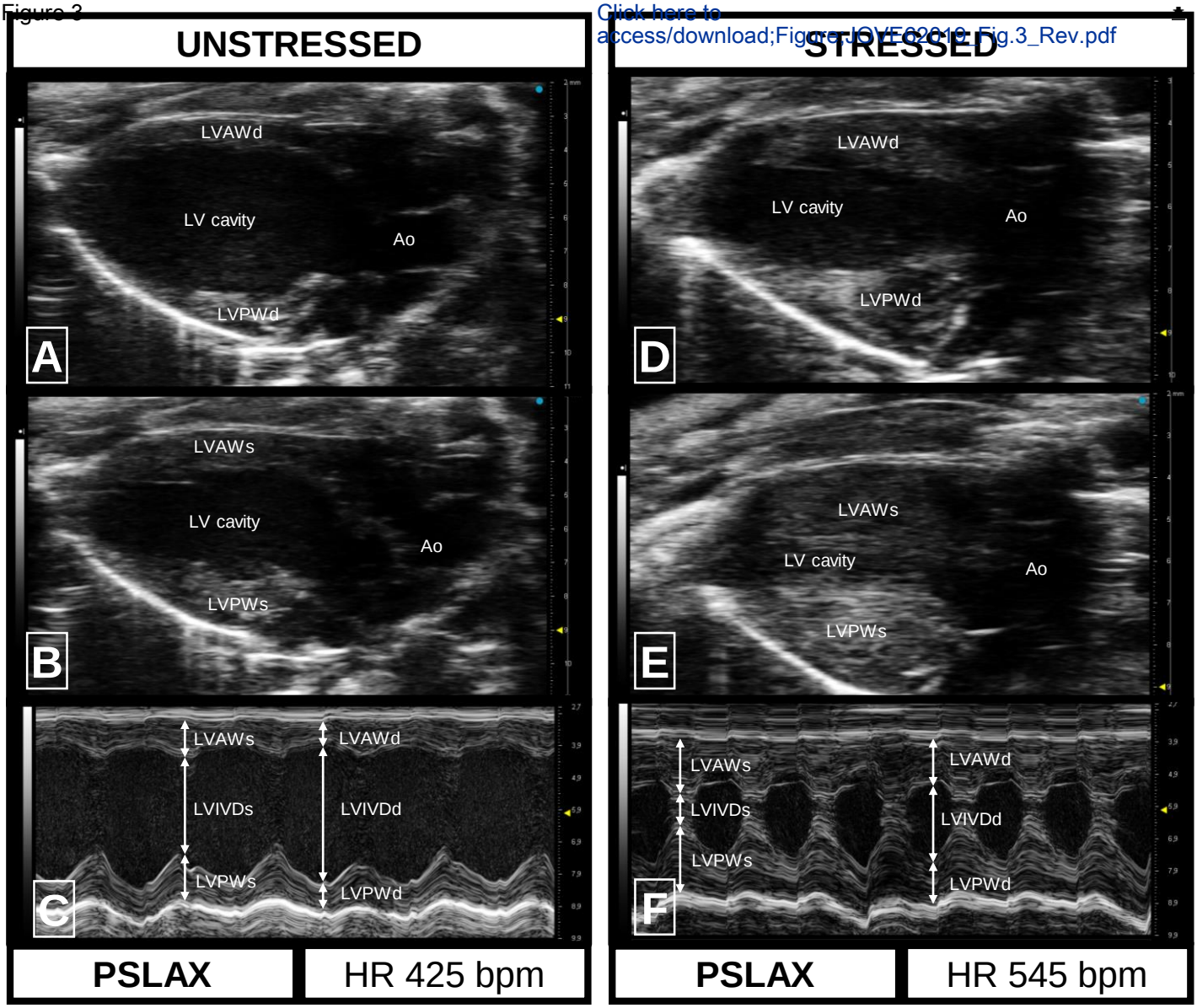
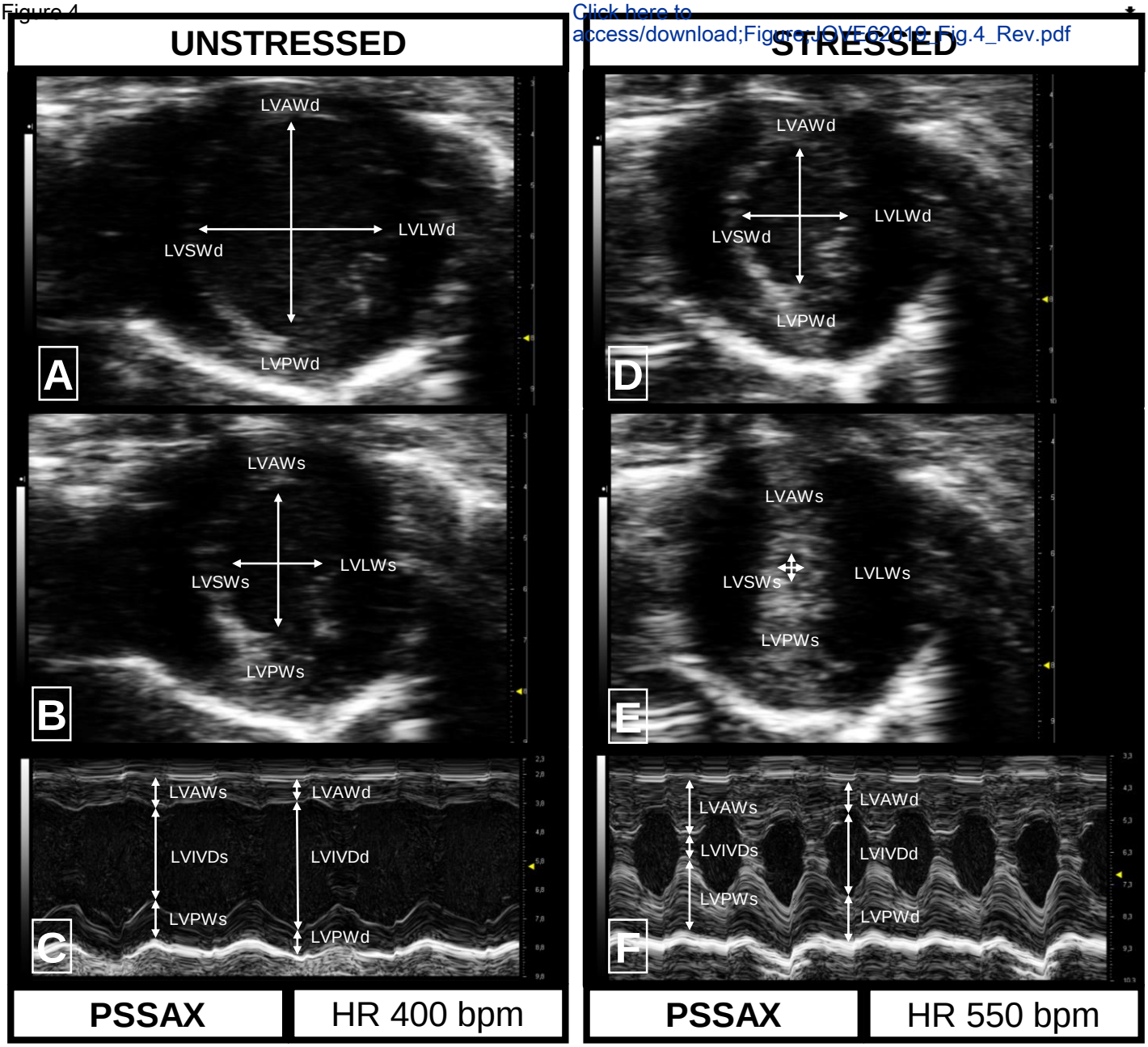


Figure 4



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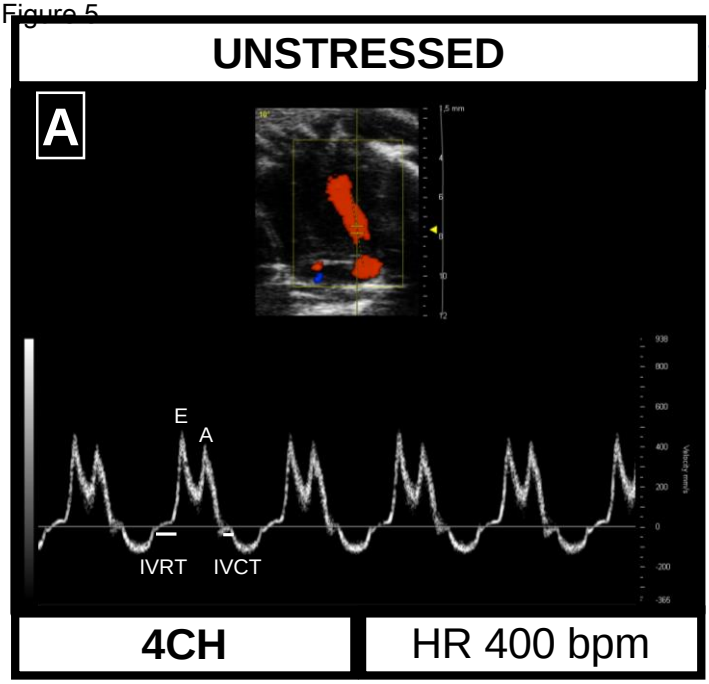

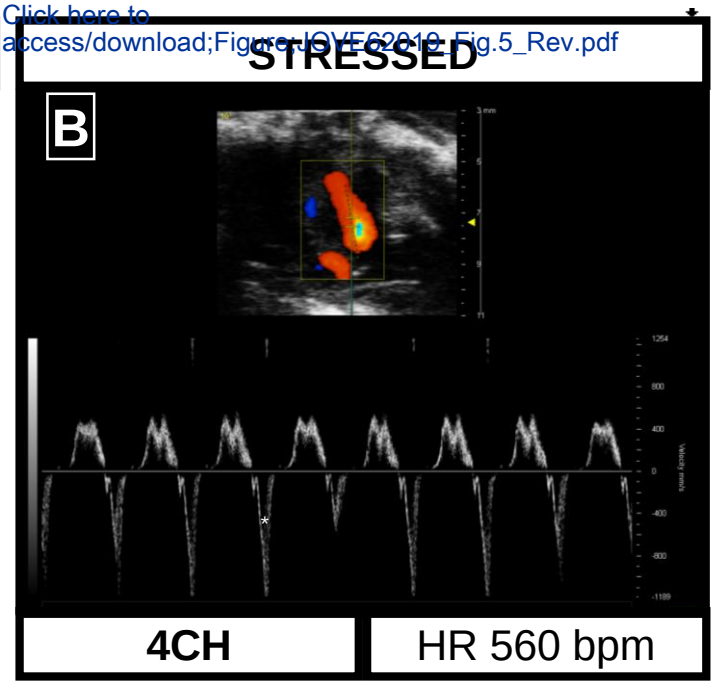

Figure 6

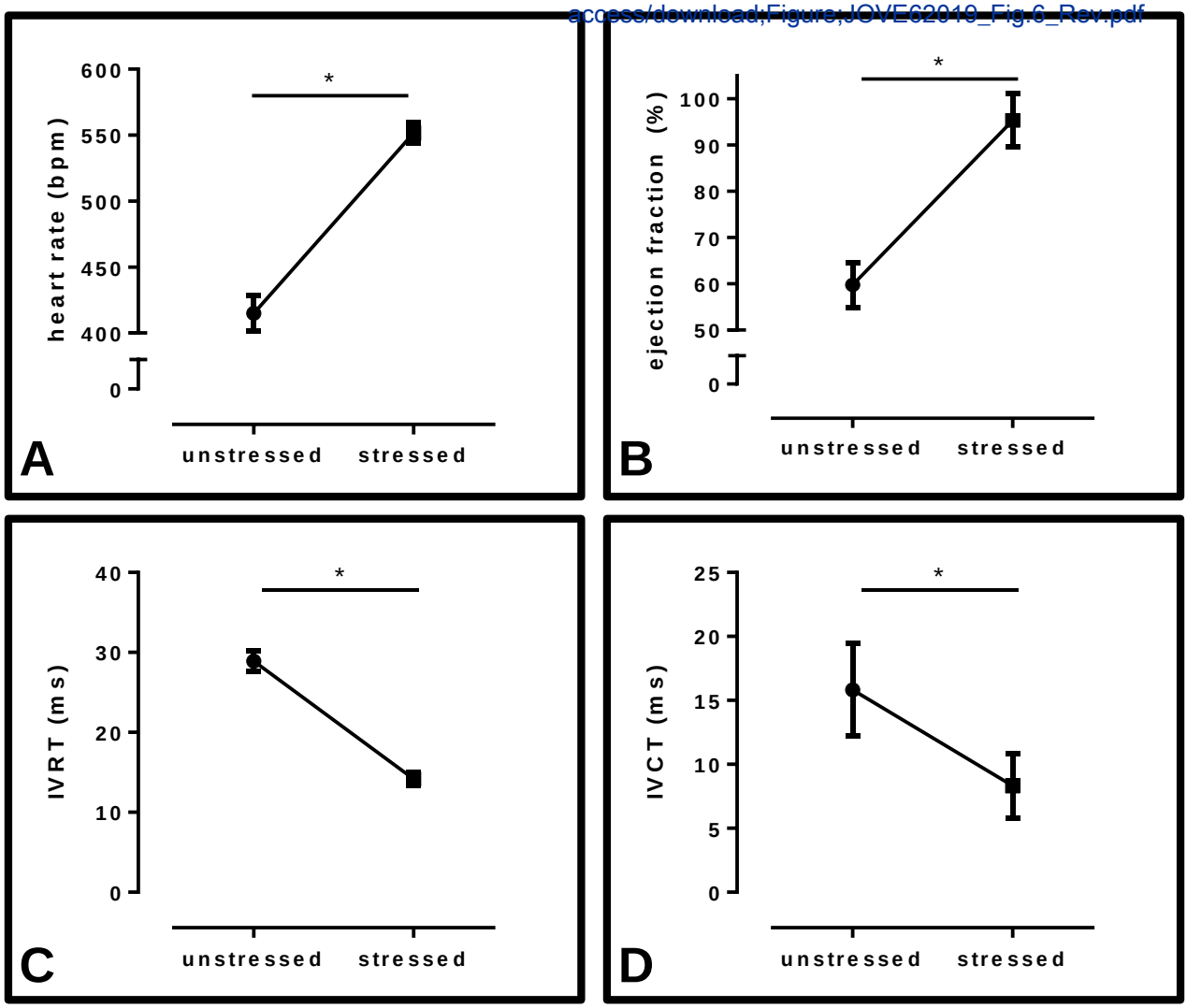

Figure 7

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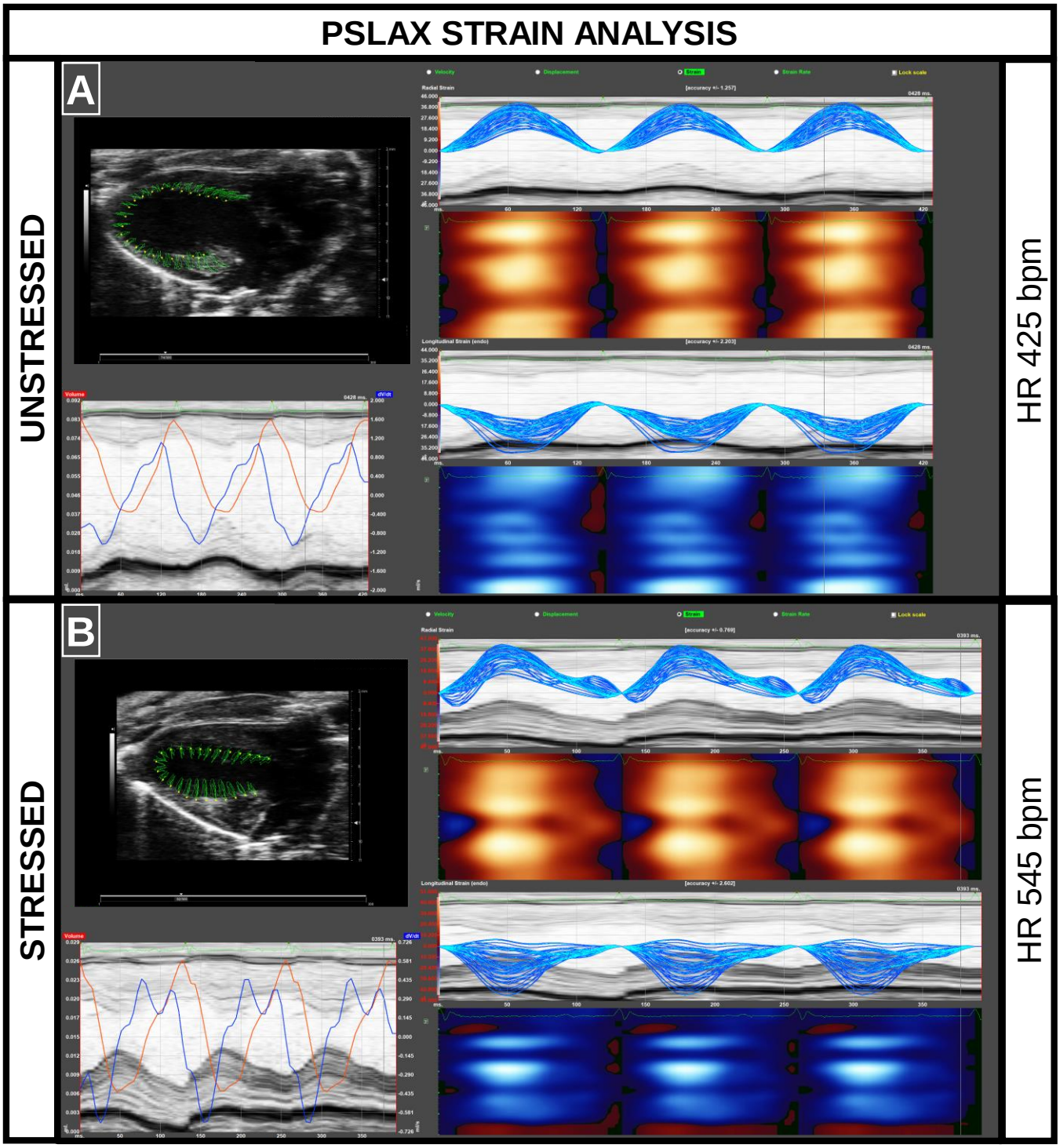

Figure 8

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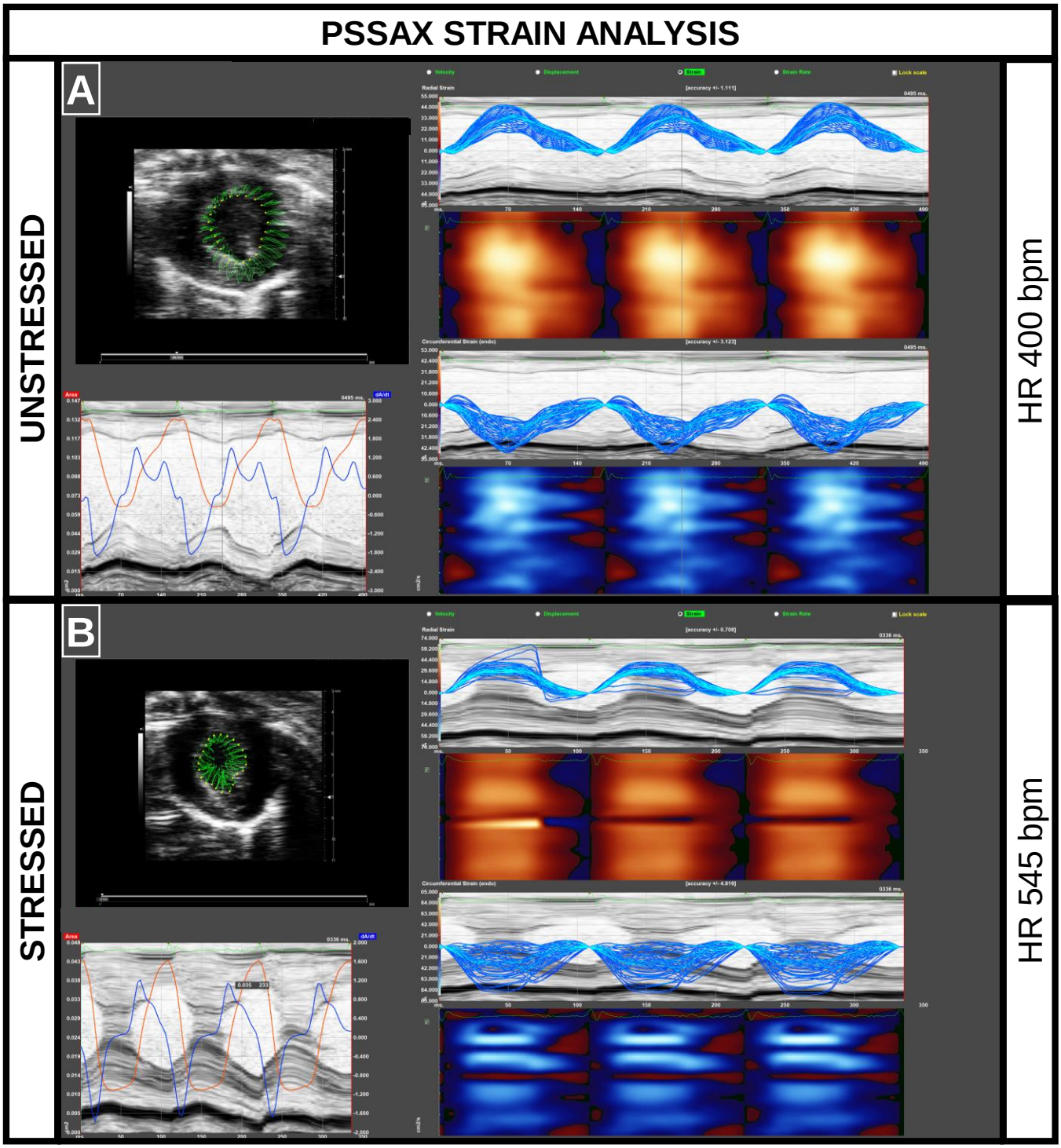

Figure 9

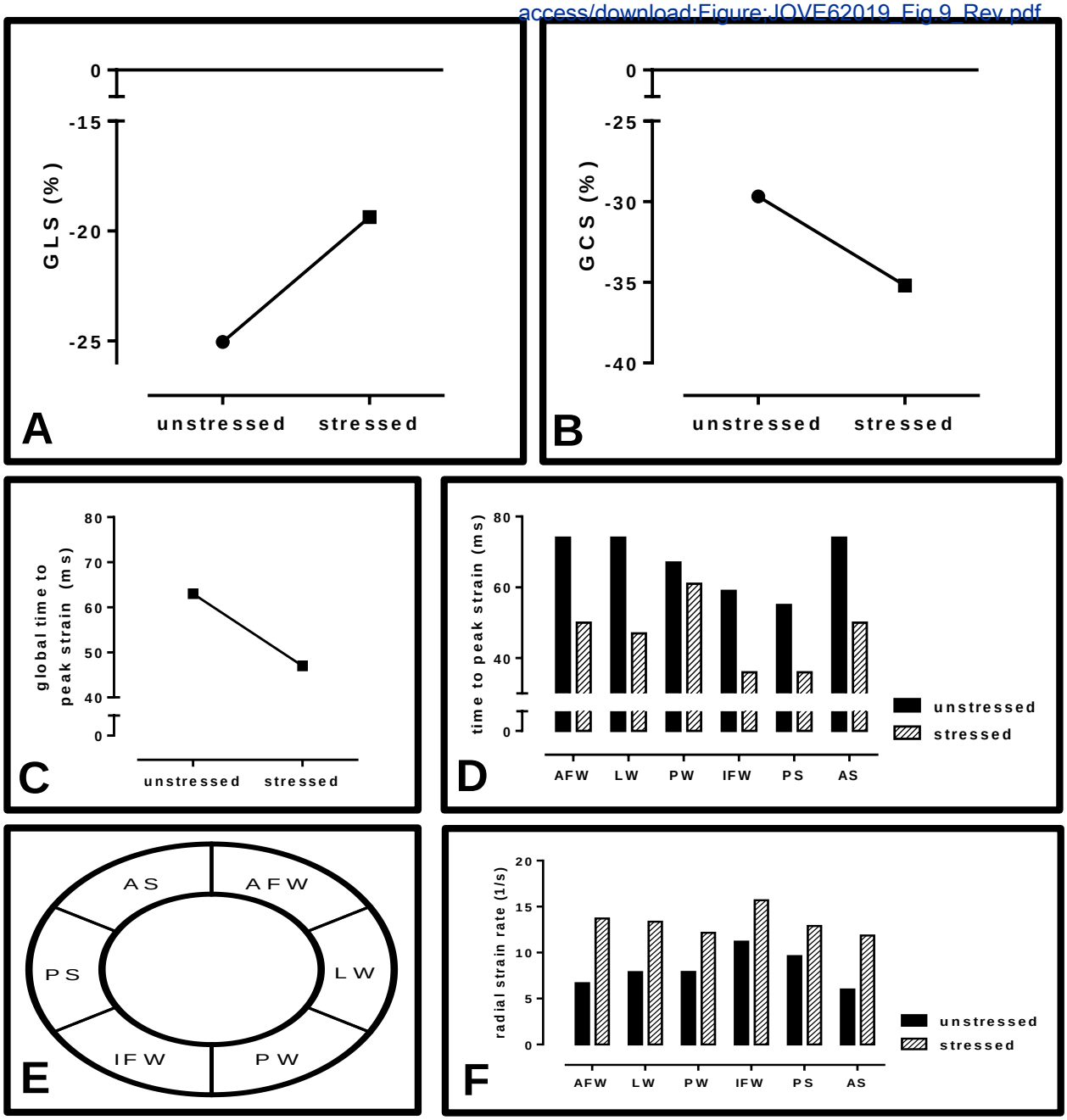

Figure 10

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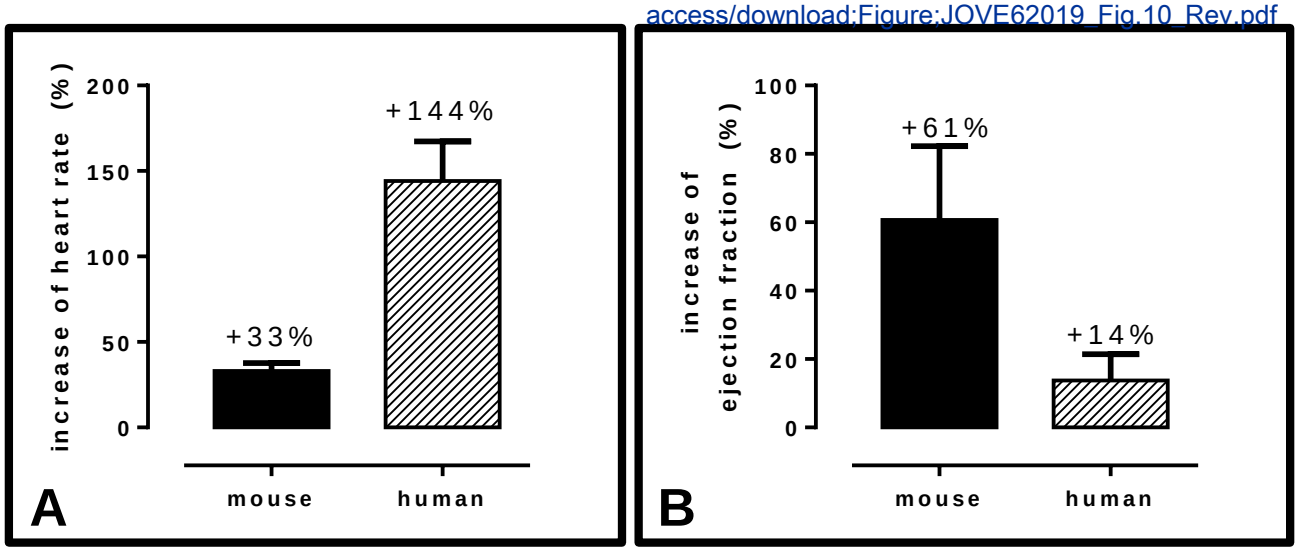

Name of Material/ Equipment	Company	Catalog Number
Activated Charcoal Filter	UNO BV	180000140
Aquasonic 100 Ultrasound Transmission Gel	Parker Laboratories	001-02
Chemical Hair removal lotion	General Supply	-
Cotton Swaps	General Supply	-
ddH2O	General Supply	-
Dobutamine	Carinopharm	71685.00.00
Flowmeter for laboratory animal anesthesia	UNO BV	SF3
Gas Exhaust Unit	UNO BV	-
Heating Lamp	Philips	-
Induction Box	UNO BV	-
Medical Sharps Container	BD	305626
MX400 ultrasound transducer (20-46 Mhz)	VisualSonics	MX400
Octenisept disinfectant	Schuelke	173711
Omnican F syringe with needle 1ml	B. Braun	9161502S
Paper Towels	General Supply	-
Signacreme Electrode Cream	Parker Laboratories	017-05
Standard Gauze Pads	BeeSana Meditrade	4852728
Thermasonic Gel Warmer	Parker Laboratories	82-03-20 CE
Transpore Tape	3M	1527NP-0
Vaporizer Sigma Delta	UNO BV	-
Vevo 3100 high-frequency preclinical ultrasound imaging system	VisualSonics	Vevo3100

Vevo Imaging Station with integrated rail system, heated platform and physiological monitoring unit	VisualSonics	-
VevoLab Analysis Software	VisualSonics	Vers. 3.2.5

Comments/Description

http://www.unobv.com/Rest%20Gas%20Filters.html

https://www.parkerlabs.com/aquasonic-100.asp

https://www.carinopharm.de/stammsortiment/#103

http://www.unobv.com/Flowmeters.html
http://www.unobv.com/Gas%20Exhaust%20Unit.html

http://www.unobv.com/Induction%20box.html
https://legacy.bd.com/europe/safety/de/products/sharps/

https://www.visualsonics.com/product/transducers/mx-series-transducers
https://www.schuelke.com/de-de/produkte/octenisept.php

https://www.bbraun.de/de/products/b60/omnican-f.html

https://www.parkerlabs.com/Signacreme.asp

https://www.meditrade.de/de/wundversorgung/verbandstoffe/beesana-mullkompresse/

https://www.parkerlabs.com/thermasonic_apta_sbp.asp
https://www.3mdeutschland.de/3M/de_DE/unternehmen-de/produkte/~/3M-Transpore-Fixierpflaster/
http://www.unobv.com/Vaporizers.html

https://www.visualsonics.com/product/imaging-systems/vevo-3100 * required software package: Cardiovascular package; B-mode, M-mode, pul

https://www.visualsonics.com/product/accessories/imaging-stations

https://www.visualsonics.com/product/software/vevo-lab *required software package: Vevo Strain, LV analysis

sed-wave doppler mode

Rebuttal relating to the editorial and reviewer comments regarding

Revealing subtle changes in cardiac function using transthoracic dobutamine stress echocardiography in mice

by Settelmeier *et al.*

MS: JoVE62019

Dear Dr. Nguyen,

We are pleased to resubmit our revised manuscript entitled “Revealing subtle changes in cardiac function using transthoracic dobutamine stress echocardiography in mice” to be considered for publication in the Journal of Visualized Experiments.

We thank you for the editorial advice and revised our manuscript accordingly. We further addressed the valuable comments raised by the reviewers. We have tracked the changes within the revised manuscript for identification of all edits. We honestly hope that with the revised version of our manuscript other investigators may find our protocol useful to reveal subtle changes within their experimental setup using dobutamine stress echocardiography.

Thank you for considering our submission.

Yours sincerely,

Ulrike B. Hendgen-Cotta
- in behalf of all authors -

Rebuttal relating to the editorial and reviewer comments regarding

Revealing subtle changes in cardiac function using transthoracic dobutamine stress echocardiography in mice
by Settelmeier *et al.*

MS: JoVE62019

We would like to express our thanks to the editor and the referees for the thorough evaluation of our manuscript and their important comments. We have carefully considered their recommendations and incorporated the changes proposed in the manuscript.

In the following we respond in detail to the questions

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

We thank the editor for the opportunity to proofread the manuscript and corrected spelling and grammar issues. We checked all abbreviations and added definitions when necessary.

2. Unfortunately, there are sections of the manuscript that show overlap with previously published work. Please revise the following lines: the first sentence of the second paragraph of the introduction

We deeply apologize for this unintended overlap with previous published work and changed the sentence accordingly: "Since the symptoms are unspecific and more than one third of patients with the clinical syndrome of heart failure may actually not suffer from heart failure, it is important to find an objective echocardiographic correlate for the patient's clinical presentation (p. 2, ll. 11-13)

3. a) Please specify what happens to the animals after the experiments. If euthanized, please specify the method of euthanasia without highlighting it.

Due to the non-invasive nature of the presented ultrasound stress echocardiography protocol, the experiment does not require euthanization of the animals used. Once completed, the animals will wake up easily and will be returned to their cage. We added the information as NOTE accordingly (Step 5.3):

"NOTE: Due to the non-final character of this technique, the animal may stay within the experiment in accordance with all relevant regulations." (p. 7, ll. 13-15)

b) For survival strategies, discuss post-surgical treatment of animal, including recovery conditions and treatment for post-surgical pain.

The presented protocol is a non-invasive painless ultrasound evaluation; therefore, no surgery is performed on the animal within our protocol and there is no need for treatment of post-surgical pain. Nevertheless, recovery conditions are essential for every organism being under anesthesia. Therefore, we stressed the need to observe the animal closely during recovery from anesthesia within a separated heated cage and added details to the "protocol" section (Step 5.3).

"Place the animal on a paper towel in a separated heated cage during the wake-up period. Observe the animal closely. It must not be left unattended until it has regained sufficient consciousness to maintain sternal recumbency." (p. 7, ll. 10-12)

c) Discuss maintenance of sterile conditions during survival surgery.

Because our approach does not include surgical interventions (3b), dedicated sterile surgery conditions are not needed. Nevertheless, it is important to work in a safe and clean environment and to use only sterile single-use sterile injection equipment for the dobutamine injection to prevent from infections, especially in long-term experiments. We added this to our protocol in Step 1.3 and 4.3:

"Clean all instruments including the platform with a disinfectant wipe." (p. 3, l. 20)

"NOTE: Always use single-use sterile injection needles for every animal to prevent from infections." (p. 6, l- 13)

d) Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.

We clarified this in our protocol (step 5.3.):

"It must not be left unattended until it has regained sufficient consciousness to maintain sternal recumbency." (p. 7, ll. 11-12)

e) Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.

We clarified this important point in our protocol (step 5.3.):

"Once awake and fully recovered, transfer the animal to its cage." (p. 7, ll. 12-13)

4. After including a one line space between each protocol step, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.

We have added the one line space between the protocol steps and highlighted the needed text passages for the inclusion in the protocol section of the video.

5. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I.,

LastName, F.I. Article Title. Source. Volume (Issue), FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Do not abbreviate journal names.

We have assured that the references appear according to the standards of JoVE.

Reviewers' comments:

Reviewer #1: Manuscript Summary: In this manuscript, the author offered a detailed and delicate description of the dobutamine stress echocardiography (DSE) in mice and compared the result with human DSE. However, several issues should be addressed.

We thank the reviewer for the detailed feedback and after incorporating the reviewer's remarks, we hope the manuscript is now acceptable.

Major Concerns:

1. The author used only one dose of dobutamine for DSE, and only recorded the imaging after reached the target HR. Based on the dose-depended nature of dobutamine, a different dose of dobutamine (from low to high) could be used for the DSE. What is more, continuous recording of echocardiographic images is also necessary to observe the changes of HR, EF and LV wall movement during DSE, as HR and cardiac contractility has different timing of responses to dobutamine stimulation.

We thank the reviewer for raising this point. We absolutely agree, when dobutamine stress echocardiography is used on human patients, a step-up protocol of dobutamine doses is used until a predefined stop criterion (dyspnea, chest pain etc.) or the final (sub)maximal load for the patient is reached with the aim to search for intra- and/or differences in diagnostics. When applied to identical mice within an experimental group, we do not search for interindividual differences but would rather like to achieve a comparable and reproducible identical (sub)maximal load to reveal subtle changes in the cardiac function within our experimental group. This aim is reached by administering one and the same in pre-experiments titrated dobutamine dose which reaches the significant increase in cardiac functional parameters.

We agree with the reviewer's point that there may be different timings in response to dobutamine stimulation. We here aimed for a protocol providing a rapid assessment of unstressed and stressed mice hearts which can be applied repetitively in long-term experiments as well. Therefore, we suggest the analysis and comparison at the point of (sub)maximal load. Of course, continuous recording of the echocardiographic images is necessary to observe changes and to define the plateau of (sub)maximal load for later analysis.

We concretized our manuscript as follows:

i) Importance of dose-finding in pre-experiments: Step 4.3. "The dobutamine susceptibility and the (sub)maximal load may vary with the mouse strain and may be dependent on the experimental setup and should be defined in pre-experiments." (p. 6, ll. 17-20)

ii) Dobutamin administration: discussion "We here decided for i.p. injection of 5 µg/g body weight because it showed a significant, robust, reproducible and transient increase in cardiac functional parameters reaching the (sub)maximal load in male C57BL6/6JRj mice used. Because the protocol is, unlike to human patients, not searching for interindividual differences within the experimental mice group, the same (sub)maximal load should be reached in every mouse examined requiring the same application dose in every animal. At this point we would like to point out that dose-response testing until a significant, robust, reproducible and transient increase in cardiac functional parameters is reached should be done dependent on the mice strain and the experimental setting beforehand. " (p. 11, ll. 33-41)

iii) Continuous recording of echocardiographic images during heart rate increase: step 4.3 "Record echocardiographic images until the target heart rate is reached and use the increase of the heart rate for later analysis." (p. 5, ll. 14-15)

2. Figure 10 showed that during DSE, humans showed a 137% increase of HR and 14% increase in EF, while mice had only a 33% increase in HR in mice but a 61% increase in EF. So, based one that, the author stated that the effect of dobutamine is rather the inotropic than the chronotropic effect in mice compared to the human. This statement is not valid and overinterpreting and should be removed as the cited study (ref 12) for human beings was performed on patients, not really normal human beings. So the comparison is not fair at all. In addition, there is a dose issue. Dobutamine has the dose-depended effect in human (low dose showed inotropic effect, while high dose showed chronotropic effect). We usually give patients different doses of dobutamine for DSE (from 5 µg/kg/min to 40 µg/kg/min), while one dose was mentioned in this manuscript (5 µg/g BW). When making comparisons between mice and humans, the dose difference should be considered.

We apologize that the initial version of the manuscript has given wrong impressions. Our intention was not to do an interspecies comparison between mice and human but rather to point out differences in translational approaches. The aim was to sensitize the reader going from bed to bench not to expect human heart rate increases in mice and vice versa. We agree with the reviewer, that the statement was not valid and overinterpreting.

First, we removed the reference referring to human patients and replaced both heart rate increases and the reference itself with data from healthy volunteers.[1]

Second, we recalculated the heart rate increase for healthy volunteers and furthermore used measurements from physiological daily routine stress echocardiograms and observed an average increase in heart rate of 144 %. We have corrected the passages, added additional literature and now hope to explain the findings in a more consistent manner.

Third, the result section has been rewritten: "The results of dobutamine administration vary between species, which is important for translational approaches. Once, (sub)maximal load is reached, mice showed an average heart rate increase of +33 % while humans showed an average heart rate increase of +144 % (**Figure 10 A**). At (sub)maximal load, mice showed an average dobutamine-induced EF increase of +61 %, humans showed an average EF increase of +14% (**Figure 10 B**)." (p. 8, ll. 29-33)

Fourth, the first section of "Between mice and human: Translational application" of the discussion has been rewritten: "As we have shown, the effects of dobutamine vary between mice and humans. Translational preclinical researchers should not expect the same increase of cardiac functional parameters they are used to in human patients or vice versa." (p. 12, ll. 14-16)

Fifth, we would like to address the dose issue. As described in the answer to 1., a step-up protocol is used in human patients which is not needed (as long as sufficient pre-experiments had been performed) in the presented approach as the measurements should be performed at the point of (sub)maximal load within the plateau phase of dobutamine induced effects. The dobutamine effects shown in Figure 10 are measured at the point of (sub)maximal load as well. Therefore, the dose differences arise from the administrative route (i.v. *vs*. i.p.), the species (human *vs.* rodent) and different dose-finding regimes (step-up protocol in humans *vs.* pre-experimental dose-titration in mice). We have added this point to our discussion: "If, however, a comparison should be performed, we suggest only to compare the points of (sub)maximal load." (p. 12, ll. 16-17)

3. The author used both conventional and sophisticated echocardiography analysis, strain analysis, to evaluate the systolic and diastolic cardiac function. More detailed strain and strain rate analyses should be presented. For example, the SD of Time to Peak (TPk), which represents the systolic synchrony, of six segments of LV should be presented. And the strain rate of LV (S wave) is another important parameter for LV systolic function, which represents the systolic rate of LV.

We thank the reviewer for this certainly important remark. While most of the preclinical studies still rely on conventional echocardiographic analysis, more advanced techniques become increasingly important. Due to the point raised by the reviewer, we added more information on TPk analysis and segmental synchrony analysis which changes after dobutamine injection for evaluation of the systolic LV function and will be of interest for researchers investigating effects of e.g. myocardial infarction in mice (revised Figure 9). Because of the instructional exemplary character of the JoVE article and video, we hope that with the revised manuscript we have found the right balance between conventional and advanced analyses. We furthermore added information and literature to the result section:

"Strain reflects the deformation of an object normalized to its original shape and size[2] which equals the length of the myocardial fiber normalized to its original length[3], strain rate represents the myocardial deformation rate. Strain can be measured in the radial, circumferential and longitudinal axes. One example is the measurement of the radial strain in PSSAX, which describes the increase of the myocardial wall thickness during the cardiac cycle and represents its deformation towards the center of the left ventricle.[4] The cardiac systolic synchrony is a measure of the systolic function of six LV segments (Figure 9 E).[5,6]" (p. 8, ll. 16-21)

4. For figure 8, "PSLAX" should be "PSSAX". And in figure 8B, the first cardiac cycle, which seemed much longer than the following two, should be excluded. Or the author should give some advice or solutions for arrhythmia. If the mice have uncorrectable arrhythmia, should the mice be excluded? Or, more than five cardiac cycles should be saved to calculate the average echo parameter (both conventional and sophisticated)?

We thank the reviewer for this important issue. In the revised version, we have now entitled Fig. 8 correctly. The intention of Fig. 8 B was to point out possible pitfalls and limitations in strain analysis of stressed hearts that may occur. This was more confusing than helpful, especially because performing calculations on precisely recognized parts of the contraction cycle and R-wave-triggered analysis will dramatically reduce the probability of incorrect measurements. Therefore, the first cardiac cycle initially displayed was – on purpose – not correctly recognized. We have revised Fig. 8 including the caption and now show a clear PSSAX stressed analysis:

"In the healthy animal, the wall signals are uniform (red/blue pattern)." (p. 9, ll. 24-25)

With clear images, the proposed acquisition of at least 3(-6) full cardiac cycles (Step 3.2.) appears sufficient. (p. 5, l. 19)

In healthy mice and examination performed in the described manner, arrhythmia should not manifest, therefore we decided not to cover arrythmia as possible interventions may interfere with experimental results. If arrythmia is part of the experimental setting (e.g. electrophysiological considerations), some analyses may not be applicable. Because this depends largely upon the experimental setting, we hope that our decision not to cover this point in detail within the methodical manuscript is understandable.

5. The author used baseline HR as 450 ± 50 bpm, and they also mentioned that "The EF in mice is depending on the heart rate, reported between 58 ± 11 % (< 450 bpm) and 71 ± 11 % (≥ 450 bpm) and changes by nearly 20 % with higher heart rates". So, in this manuscript, their baseline HR range is 400-500 bpm, that is too large. And baseline EF for different experiment groups may be different if one group has baseline HR for 400-450 bpm, while another group has baseline HR for 450-500 bpm.

We agree with the reviewer's position that an adequate baseline heart rate is essential for comparison of experimental results and therefore proposed a narrow heart rate corridor. In our manuscript, we show data within a heart rate range of 400-450 bpm (Fig. 3: 425 bpm; Fig. 4: 400 bpm; Fig. 5: 400 bpm; Fig. 7: 425 bpm; Fig. 7: 400 bpm) to secure comparability.

Nevertheless, maintaining a stable heart rate in the daily routine is a challenging issue: In literature, a variety of target heart rate ranges are proposed: Respress and Wehrens proposed 450 ± 50 bpm[7], Gao et al. "generally maintained at 400-500 beats per minute" and stated a "variation of HR within 100 bpm for a strain/set of experiments […] acceptable"[8]. Lindsey et al. mentioned in detail: "A heart rate of >400 beats/min is advised to be within the physiological range of murine heart rate under anesthesia. […] While it is important to sustain a high heart rate during assessment of cardiac function in rodents, heart rates of >650 beats/min suggest activation of the autonomic nervous system, and, therefore, results with heart rates of <400 and >650 beats/min should be interpreted with caution."[9] Of note, Zacchigna et al. defined >450 bpm as physiological heart rate and defined <450 bpm as (slightly) depressed by anesthesia[2], whereas the decided strict cut-off of 450 bpm seems less practical.

We here believe that a target heart rate of 400-450 bmp or slightly higher (intentionally represented by the term 450±50 bpm) represents a feasible and pragmatic approach which can be usually be reached as long as the target heart-rate corridor within one mice during the procedure and between another group of mice remains narrow within 50 bpm.

For clarification and to avoid misunderstanding, we adjusted our manuscript according to the point raised by the reviewer (step 2.6., step 4.1):

"To protect the animal from stress during the procedure, check the adequate depth of sedation by maintaining the heart rate range at 400-450 bpm. A variation of 50 bpm within the range is acceptable." (p. 4, ll. 22-23)

Make sure your starting heart rate is stable in the range of 400-450 bmp. The ECG will be recorded and saved together with and on the acquired images." (p. 6, ll. 5-6)

We furthermore added the point of inter-group-comparability to the discussion (Anesthesia and heart rate):

"At this point, it is important to use the same heart rate corridor within one group of mice and compared to another group of mice." (p. 11, ll. 25-26)

6. How did the author definite basal, midventricular and apical level of LV short-axis view?

An appropriate view of the multi-plane parasternal short-axis view is indispensable. We performed our study according to the position paper of the ESC working group on myocardial infarction.[2] We scrolled along the long axis to set the most basal and the most apical sections in which the LV chamber is still

visible in the whole cardiac cycle without including the left atrium at the base. The midventricular level is represented by the intermediate position at the level of the papillary muscles. For clarification and reproducibility, we have added the information to step 3.3.:

"[…] To define the most basal and the most apical view, we recommend scrolling along the long axis to the most distant points, where the full cardiac cycle of the LV chamber is still visible. Take images on the midventricular level approximately in an intermediate position at the level of the papillary muscles […]" (p. 5, ll. 24-27)

Minor concerns:

7. Is there any video provided?

Right now, there is no video provided. The video production will be scheduled, once the final revised manuscript is accepted. So, we hope that the revised manuscript is acceptable to provide a corresponding video soon.

8. At line 197, safe should be saved.

We have corrected the spelling mistake.

9. Please add an explanation of Bowditch effect or staircase phenomenon in the introduction.

We have added an explanation of the frequency-dependent positive inotropic activation to the introduction:

"Partly responsible for this mechanism is the Bowditch effect or staircase phenomenon, a frequency-dependent calcium-mediated positive-inotropic cardiac response […]". (p. 2, ll. 36-37)

Reviewer #2:
Manuscript Summary:
This manuscript describes conducting stress echocardiography in anesthetized mice using dobutamine. The protocol is explained in detail in stepwise manner along with the cautions. The results and discussion are clear. Finally, comparison of human and mice is helpful to check the applicability of this method to translational research.

We thank the reviewer for his differentiated review and hope, the revised manuscript is now suitable for consideration for publication.

Major Concerns:
None

Minor Concerns:

The authors may need to discuss other catecholaminergic drugs such as isoproterenol-induced stress echocardiography.

We thank the reviewer for this helpful recommendation, as the discussion of various catecholaminergic drugs has been far too limited. We therefore restructured the entire paragraph on the administration of dobutamine, including a discussion of isoproterenol for stress echocardiography in mice:

"As predominantly selective β_1-adrenergic drug, dobutamine is often chosen due to its cardiac inotropic and chronotropic effects with minimal direct effect on the blood pressure in preclinical studies.[9] As an alternative to dobutamine, the administration of isoproterenol is possible. Isoproterenol is a non-selective β-adrenergic agonist with effects on both β_1- and β_2-adrenergic receptors, which – in contrast to dobutamine - causes a lowering of the mean arterial pressure after application due to vasodilatation. It leads to cardiac hypertrophy and fibrosis and is used to mimic a stress-induced cardiac injury in a rather acute and as heart failure model in a rather chronic administration, so that it is somewhat inferior to dobutamine when repeated stress echocardiography is planned in a longitudinal animal experiment. Nevertheless, the comparison of both substances did not show significant differences in inotropic and lusitropic effects in healthy mice. Overall, dobutamine is more commonly used for stress testing in the clinical setting, while isoproterenol is seen in the experimental setting." (p. 122, ll. 6-17)

Reviewer #3:
Manuscript Summary:
In this communication, the authors describe a protocol for transthoracic echocardiography in mice with administration of dobutamine to assess cardiac function. The representative results indicate differences between mice and humans in the chronotropic and inotropic effects of dobutamine. The detailed setup description provides the field with a clear methodology to assess cardiac function in murine models of cardiac disease. The authors provide detailed instructions, considering important points for animal welfare during imaging. There are minor concerns that should be addressed before the manuscript is considered for publication.

We are thankful for the reviewer's comments, especially for highlighting the important points for animal welfare. We hope that after incorporating the reviewer's remarks the point by point revised manuscript is now acceptable.

Major Concerns:
*** The authors note that the analysis software is not able to distinguish systole from diastole in some stressed hearts (Fig 8B). Can the authors clarify how they analyze the data in this instance? How shall other investigators modify their analysis methods in such instances?**

We highly agree with this point raised by the reviewer. The intention of Fig. 8 B was to point out possible pitfalls and limitations in strain analysis of stressed hearts that may occur. This was more confusing than helpful, especially because performing calculations on precisely recognized parts of the contraction cycle and R-wave-triggered analysis will dramatically reduce the probability of incorrect measurements for other researchers. Therefore, the first cardiac cycle initially displayed was – on purpose – not correctly recognized. Since the single steps depend heavily on the type of software used, we are unfortunately unable to provide step-by-step instructions how to use the various strain analysis software. In the revised version of Fig. 8 B, we now use and show clear recognized cardiac cycles that have been analyzed by the software easily.

We have added the importance of clear images for reliable measurements at the end of the discussion paragraph "evaluation of cardiac function":

"Attention in stress testing strain analysis is needed, as analysis of high heart rates and significantly induced cardiac function parameters depends on calculations performed with the accurately recognized parts of the contraction cycle." (p. 12, ll. 40-43)

*** Based on the misinterpretation of the stressed state of the software in Fig 8B, Fig 9 appears misleading.**

Based on the revised Fig. 8, we would like to assure at this point that the measurements used for Fig. 9 were reliable performed on the basis of images with clear image quality as well as software recognition. We hope that with the revised version of our manuscript, there is no misdirection concerning the stressed heart strain analysis anymore and that other investigators will find our protocol useful to reveal subtle changes within their experimental setup using dobutamine stress echocardiography:

Figure 9: Changes in strain parameters in unstressed and stressed animals. A Decrease of global longitudinal strain. **B** increase of global circumferential strain. **C** Reduced global time to peak strain. **D** Reduced time to peak strain across all six segments. **E** Schematic overview of anatomical segments in

short axis view. **F** Increased radial strain rate after dobutamine administration. As values are software-dependent and referent values in mice are currently not present, the values have to be seen individually for each experimental setup. The graphs represent the result of Figure 7 and 8. AFW = anterior free wall; LW = lateral wall; PW = posterior wall; IFW = inferior free wall; PS = posterior septum; AS = anterior septum. (p. 10, ll. 27-34)

Minor Concerns:
*** Line 57: perhaps the authors meant "clinical symptoms" instead of "clinical syndrome"**

In this case, the intended clinical syndrome of heart failure refers to “syndrome” as the “combination of (unspecific) symptoms”. Re-reading the sentence, the use of “heart failure symptoms”, “clinical syndrome of heart failure” and “suffer from heart failure” appears pleonastic. Therefore, we changed the wording to avoid pleonasms:

“Since the symptoms are unspecific and more than one third of patients with the clinical syndrome of heart failure may actually not suffer from heart failure […]” (p. 22, ll. 11-13)

*** Step 2.8 has a typo - "lightning" should be "lighting"**

We have corrected the spelling mistake.

*** Line 189 "careful not to apply to much pressure" should be "too much pressure"**

We have corrected the spelling mistake.

*** Step 4.2 typo - "Safe" should be "save"**

We have corrected the spelling mistake.

*** The authors indicate that images with comparable heart rate increase should be analyzed. Please detail what steps are taken to record HR with the image acquisitions.**

Usually, the ECG is recorded and saved together with the ultrasound images. This is, as the reviewer pointed out, essential for later image analysis. We added detailed information on the steps, especially in step 4.1., what to do if the ECG signal is of poor quality:

Step 2.5: “[…]The ECG is used to record the heart rate during image acquisition. “ (p. 2, ll. 16-17)

Step 4.1.: “The ECG will be recorded and saved together with and on the acquired images. Make sure that the ECG signal is clear. Otherwise, try to re-tape all four limbs until a clear ECG signal is displayed.” (p. 6, ll. 6-8)

*** What is the purpose of Fig 1B image of a syringe?**

Our intention was to show the syringe as reminder to prepare the dobutamine injection beforehand. We agree, that for the benefit of the reader, a larger view of the small animal echocardiographic workplace (former Fig. 1A) is much more important than a picture of a syringe. Therefore, we removed the picture and hope to provide a more clearly arranged figure.

*** The introduction notes dobutamine produces an average heart rate increase of 37% in humans (line 75), whereas the results section notes an average 137% increase in human HR (line 279). Please address this contradiction.**

We apologize for the mistake and the resulting inconsistency. Of course – and in accordance with the references provided – a heart rate increase of 37 % (which would correspond to an increase from 70 to 97 bpm) is not natural. Actually, an increase during (sub)maximal load of about 150±10 % in healthy volunteers[1] is reported in the literature, which seems comparable to patients[10]. In the results section, we furthermore used measurements from physiological daily routine stress echocardiograms and observed an average increase in heart rate of 137%. We have corrected the passages, added additional literature and now hope to be able to explain the findings more consistently.

Introduction: "The human target heart rate in dobutamine stress echocardiography is [(220-age)x 0.85], resulting in an average heart rate increase of about 150±10 % in healthy volunteers." (p. 1, ll. 29-31)

*** Discussion of dobutamine administration - please note that dobutamine infusion can be accomplished with a tail vein catheter and infusion pump, as described in Castle et al 2019 (PMID: 30291650)**

We thank the reviewer for this valuable advice. We have therefore restructured the entire paragraph on dobutamine administration, including a discussion of i.v. referring to Castle et al. as an administrative route, while discussing our choice of i.p. injection. Of course, the ideal administrative route may depend on the setting of the experiment.

"I.p. injection is a feasible administrative route as i.v. injection in mice requires a higher effort. Furthermore, assuming the comparable peritoneal resorption rate of dobutamine in mice, the i.p. injection approach avoids technical barriers and inaccuracies in i.v. tail vein injection, especially when serial testing is performed in longitudinal experiments, which would require several tail vein injections of previously punctured vessels. Nevertheless, similar to human procedures, an i.v. application approach could also be adapted[8,11,12]. Because of different pharmacodynamics and pharmacokinetics, this may require an additional dose-response testing in advance." (p. 11, ll. 42-44, p. 12. ll. 1-5)

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