Physical disturbance induces heart stop in mangrove killifish, *Kryptolebias marmoratus* embryos

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Summary

The mangrove killifish, *Krypotlebias marmoratus*, is an intriguing vertebrate species, known for its unusual sexual systems. It is one of two known self-fertilizing simultaneous hermaphroditic vertebrates, alongside the related *Kryptolebias ocellatus*[1,2]. While individuals can be born as primary males, this is rare in the wild and most are born as hermaphrodites[3], around 60% then turning into secondary males at 3-4 years of age[4]. *K. marmoratus* is exceptionally tolerant of harsh and variable environmental conditions. This includes emersion as well as widely ranging salinities and temperatures[5–7]. Embryos can enter diapause, a state of arrested development, when conditions are unfavourable[8,9].

Intentional heart stopping by animals is an exceptionally rare phenomenon. It may occur in species such as the sea iguana *Amblyrhynchus cristatus* and Komodo dragon *Varanus komodoensis*[10,11], although detailed studies on this behaviour in these species are lacking. Additionally, stopping or significantly reducing the heart rate is known to occur in some animals when in a state of dormancy such as hibernation or diapause[12,13]. We have found a highly unique ability of *K. marmoratus* embryos to stop their heartbeat in response to subtle

physical disturbances and fully recover. This study investigates this behaviour for the first time. This ability begins in the later stages of embryonic development at 9dpf. Recovery from heart stopping changes throughout development and the behaviour is retained during diapause. These results provide new insight into adaptive responses to environmental stressors and contribute to establishing this species as a model organism.

Results

Heart stopping and recovery

Heartbeats in *K. marmoratus* embryos start at 3 days post fertilization (dpf), as previously reported[14]. At 7 and 8 dpf, heartbeats were regular (Fig.1 & Supp. Fig. 1). Heart stopping in response to physical disturbance (HSPD) abilities began at around 9dpf (Fig. 3). Although the heartbeat ceased, other movements (such as that of the eyes, mouth and gills) did not stop, showing that HSPD does not cause complete paralysis of the body (Supp. Video 1). Embryos were highly sensitive, for example, gently touching the embryo with the tip of a tweezer induced HSPD (Supp. Video 2). At 9dpf, stops were short. The time to fully recover was 10-15 seconds (Fig. 2B). From 9dpf to 13dpf, lengths of heart stops and the recovery period increased. Around 13dpf, embryos can either hatch, or enter diapause[14]. Most embryos in our experiments entered diapause, prolonging hatching. This may have been related to water change frequencies. The HSPD ability was retained during diapause. However, once embryos entered diapause (~13dpf), lengths of heart stops and recovery periods became much more variable, with no clear pattern (Supp. fig. 1). Stops sometimes exceeded 60 seconds, (an example of this is in Supp. Video 2). No significant difference was found in heart rates between each age of embryos (7dpf-25dpf) ($p=0.108$) or comparing developing embryos (7-12dpf, average heart rate 55.9bpm, ranging from 34.2-70.2bpm) to diapause embryos (13-25dpf average heart rates 53.1bpm, ranging from 31.8-70.2bmp) ($p=0.3354$).

Heart stopping and restarting occurs in the sinus venosus

In *K. marmoratus* embryos, individual contractions of the sinus venosus, atrium and ventricle were clearly distinguishable (Fig. 3A, B) and could be measured independently (Fig. 3C-G). In HSPD, we found that the last beat always ended with contraction of the ventricle. The first beat after HSPD consistently began with contraction of the sinus venosus. These results indicate that the order of contractions is always sinus venosus, atrium, ventricle. The time between each chamber contracting remained regular even during the recovery period after HSPD (Fig. 3). Before HSPD, the average time after contraction of the sinus venosus for atrial and ventricular contractions were 0.16s and 0.58s, respectively. After HSPD, these were 0.17s and 0.57s. Together, these suggest the heart stop is always initiated by the sinus venosus. During HSPD, the sinus venosus and atrium remained filled with blood (as in diastole phase). Sometimes there was passive flow into the ventricle, allowing clear visualization of all chambers (Fig. 3). We found that when HSPD occurs, the ventricle was the last chamber to contract.

Effect of anesthetic on heart stopping

Tricaine blocks sodium ion channels in nerve cells, preventing initiation or transmission of signals. To determine whether HSPD was autonomous to the heart tissue or regulated by the nervous system, the effect of tricaine was tested on both developing and diapausing embryos. In all cases, tricaine-treated embryos lost HSPD abilities (Fig. 4). This suggests that HSPD is not autonomous in the heart but is mediated by a signal from the nervous system. Heart rates did not seem to be affected by tricaine treatment. The average heart rate of tricaine treated embryos was 61.0bpm, ranging from 51.4-78.0 bpm.**Discussion**

HSPD as a part of embryonic developmental process occurring before diapause/hatching

We have discovered a peculiar ability of HSPD in mangrove killifish embryos that has not been found in any other fish species so far. HSPD in mangrove killifish embryos may occur

frequently in the wild, considering cause can be attributed to even very slight physical disturbance.

The HSPD ability was consistently acquired around 9dpf, with all embryos showing HSPD abilities by 10dpf. When embryos reach 13dpf, some embryos start hatching (Mourabit et al. 2014). Therefore, from14dpf onward, when embryos did not hatch, it is likely that they entered diapause. Diapause is a state of arrested development where metabolic rate is significantly reduced, used to cope with stressful conditions[9]. As metabolism is reduced, the animal must prioritize which processes or functions energy is put into. The HSPD ability was retained in diapause embryos, suggesting that it is of high importance for survival or coping with stressors.

The duration of stops consistently increased until the end of development at ~13dpf. This indicates that the acquisition and development of the HSPD ability is part of the highly synchronized embryonic developmental processes in *K. marmoratus*, and it occurs whether they enter diapause or not. In *K. marmoratus* diapause-stage embryos, heart rate recovery was highly variable and did not seem to follow the pattern seen in 9-13dpf embryos. This suggests that the development of HSPD is complete by 14dpf, and the variable “depth” of diapause may lead to variability in the level of HSPD.

During diapause, metabolic rate is generally low and stable[15] although there is wide variation between individuals. Additionally, the length of diapause is highly variable, previous studies reporting lengths ranging from 23 to 108 days[16]. Within this time there may also be pre-diapause preparatory phases or post-diapause recovery phases, during which metabolic rates can vary[9]. These factors may contribute to the observed variation in recovery time, especially if recovery is linked to metabolic rate.

Other killifish species (*Nothobrancheus furzeri* and *Austrofundulus limnaeus*) have been reported to reduce their heart beat rates during diapause, sometimes to the extent they

appear to stop completely[17,18]. However, in these species the heart stops without physical stimulation and it occurs earlier on in embryonic development. In contrast, HSPD in *K. marmoratus* is an immediate, short-lived reaction to physical stimuli. It may be possible that the mechanisms to reduce/stop the heartbeat during diapause in *N. furzeri* and *A.s limnaeus* may be exploited in the mangrove killifish to develop the ability of HSPD.

HSPD has not been reported in other fish species, suggesting that it is a unique evolutionary survival strategy to *K. marmoratus*. It is conceivable that HSPD may have two advantages for the embryos. Firstly, as mentioned above, HSPD is possibly a function associated with the diapause. By being able to control heart rate, the embryo may be able to preserve energy in diapause. The second potential advantage of HSPD may be related to predator avoidance. *K. marmoratus* often inhabit crab burrows. During dry seasons they may remain in these small spaces with limited water supplies for weeks or months with in a state of diapause. This could increase risk of predation by creatures such as small crabs. HSPD may reduce vibrations or electric pulses that could be detected by hair sensors of small crabs searching for food in these burrows. We could not test HSPD in hatched larvae and juvenile fish due to technical difficulties and UK Home Office Animal's regulations. However, we found that when we imitated the movement of a crab's claw using the tip of a pair of tweezers, this very small physical disturbance induced HSPD (Supp. video 2). Stopping the heartbeat to reduce metabolism and avoid predation may continue to be advantageous in juveniles and adults. Therefore, it would be interesting to investigate HSPD in hatched fish for future research.

Sinus venosus starts HSPD and restart of the heartbeat after HSPD

We observed that contraction of the sinus venosus was always followed by contraction of the atrium then the ventricle. When HSPD occurred, the ventricle was always the last chamber to contract and the sinus venosus was the first to contract. After HSPD, timings of these contractions of chambers within individual heartbeats remained the same as before HSPD,

despite heart rate starting slower before recovering to the original rate. In mammalian hearts, the atrioventricular (AV) node delays the electrical signal from the atrium to ventricle – fish have an analogous structure within the AV junction to fulfil this role [19]. There is also a delay between the sinus venosus and atrium contracting. In reptiles, the sinoatrial junction causes this delay[20]. Our results indicate that the key domain in which stopping and restarting occurs is the sinus venosus, and the other two chambers cannot stop or restart contraction on their own.

HSPD is mediated by the neuronal circuit

Our results suggest that the sinus venosus is the key domain of the heart determining the initiation of HSPD and release from it. In fish, pacemaker cells are located in a ring-like shape at the sinus node, initiating contraction[21]. Therefore, it seems likely that the pacemaker cells are involved in regulation of HSPD. Vertebrate hearts are myogenic, generating their own rhythm independent of the brain. Pacemaker cells within the heart itself initiate the electrical impulse. However, results from tricaine experiments suggest that the heart stopping response is also dependent on neuronal function. Tricaine blocks sodium ion channels in nerve cells, preventing them initiating or transmitting signals. Treating embryos with tricaine completely prevented HSPD. Heart rate can be modulated by the autonomic nervous system (ANS); sympathetic neurons enhance heart rate while parasympathetic neurons reduce it. HSPD may be mediated by activation of parasympathetic neurons. However, it is uncertain whether this could apply to embryos as it depends on whether the ANS is sufficiently developed at this stage. For example, medaka embryos, despite development of autonomic nervous fibers as embryos, may not be able to regulate heart rate until one month post-hatching[22]. Therefore, further research is needed to elucidate the molecular and cellular mechanisms of HSPD.

<u>Ethical declaration</u>

All experiments and methods were performed in accordance with relevant guidelines and regulations of UK Home Office with the license number PP4402521. All methods are reported in accordance with ARRIVE guidelines. All experiments and methods approved by the Animal Welfare and Ethical Review Board (AWERB) at the University of Exeter.

Resource availability

Materials Availability

No new reagents were generated in this study.

Data and Code Availability

The research data and Pixel Variance code supporting this publication are openly available from the University of Exeter's institutional repository (ORE) at https://doi.org/10.24378/exe.5506. A description of the Pixel Variance algorithm is given in reference 28.

Acknowledgements

TK is funded by NC3Rs project grant (NC/X001121/1). RH was funded by University of Exeter – Employer Engagement & Student Employment (EESE). We thank Sulayman Mourabit, Thomas Cosgrove, Rachel Osman, Clare Bushell, Ed Grinsted, Maryia Fralova and Matt Winter for their support. We also thank Aquatic Resource Centre staff for fish husbandry and to Biosciences teaching lab staff for sharing the lab space and equipment.

Author Contributions

Conceptualization TK, Data curation RH, Investigation RH, Methodology TK, ADC, RH, Writing – original draft RH, TK, Writing – review & editing RH, TK, ADC

Declaration of interests

The authors declare no competing interests.

Main-text figure legends

Figure 1. Summary of methods for the main analysis. Three embryos were placed in an agarose mould to film HSPD. A region of interest (ROI) was selected over a single chamber. Pixel velocity algorithm was performed to find contraction speed. From this, beat count was calculated, then average beat rate pre-stimulation and time to 25% recovery after HSPD.

Figure 2. Graphs showing beat rates of 3 embryos for 6 ages during embryonic development: 8, 9, 10, 11, 12 and 16dpf. Beat rate was measured in 5-second intervals over 90s movies. At 30s, the dish was tapped, inducing HSPD. Orange bars show beat rate before the stimulus at 30s, from which average pre-stimulus beat rate was calculated, indicated by the black dotted line. Blue bars show beat rate after the stimulus. Red lines mark the average time at which the beat rate reached 25% of the pre-stimulus rate. The time point indicated by the grey bar (32.5s) was not included in recovery time due to noise in data following the stimulus, caused by slight movement of embryos. Error bars show SEM.

Figure 3. Heart chambers and contraction plots. (A) Image of K. marmoratus embryo at 10dpf stage, once HSPD ability is established. SV (sinus venosus) is indicated in orange, A (atrium) in green, V (ventricle) in blue. (B) Shows arrangement of chambers, arrows indicating the direction of blood flow. (C) shows a full SAV (Sinus venosus, Atrium and Ventricle) contraction plot from a 52-second movie of an 11dpf embryo. The dish was tapped at ~11s, stimulating HSPD. Below, individual beats are highlighted: (D) a typical beat from before the stimulus, (E) the last beat before HSPD, (F) the first beat after HSPD and (G) a typical beat after the beat rate recovers. Peaks indicate contractions for each chamber. Lines are coloured according to the chamber.

Figure 4. Effects of tricaine on HSPD. Graphs showing the effect of tricaine on heart stopping. Graphs show heart rates of 3 embryos for each age measured over 5s intervals

for 90s movies, with and without tricaine treatment. Examples are shown with 9, 11, 15 and 22dpf stage embryos. At 30s, the dish was tapped. The time point indicated by the grey bar (32.5s) was not included in recovery time due to noise in data following the stimulation, causing slight movement of embryos. Bars in orange show heart rate before the stimulus, blue bars after. Without tricaine treatment, embryos stop their heart in response to the tap. When embryos are treated with tricaine they lose the ability to stop their heart. The black dotted line shows the average heart rate, calculated from the first 30s of the movie. Error bars show SEM.

STAR Methods

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Mangrove killifish source and husbandry

The mangrove killifish *Kryptolabias marmoratus* wild type Hon9 line[23] was sourced and maintained in the Aquatic Resource Centre at the University of Exeter. These fish were kept in a recirculation system under artificial photoperiods of 14 hours of light to 10 hours of darkness. All fish were hermaphrodites.

Embryo collection/incubation

For this study, the *K. marmoratus* Hon9 line[23] was used. Embryos were incubated at 26°C in 14.6ppt artificial brackish water. Water changes were made every other day. Embryos were aged according to the Mourabit *et al.,* 2011 staging reference[14]. Embryos used for this study were of ages up to 25dpf.

METHOD DETAILS

Tricaine treatment

Three embryos for each age (11dpf, 15dpf, 17dpf, 18dpf, 21dpf, 22dpf) were used for the analysis. Embryos were initially treated with 0.08% tricaine/deionized water for 30 minutes before starting filming. In some cases, this dose had no noticeable effect (probably due to their hard/thick chorion) so the dose was increased in 0.04% increments until tricaine was having an observable effect (lack of jaw and fin movement). The final doses used were 0.08% for 9dpf, 11dpf and 22dpf; 0.16% for 15dpf, 17dpf and 18dpf.

Imaging & filming heart stopping

Agar moulds were made using 1% molecular grade agarose in deionized water with a duplex cable wire. The cable wire was removed once the agar was set. For filming heart stopping, three embryos were placed in the agar mould with 14.6ppt water and placed under the microscope (Fig. 1). Embryos were left to settle for 5 minutes to allow for recovery from HSPD that may have occurred from moving embryos. Movies were 90 seconds long, at 30 seconds the dish was gently tapped with a pair of tweezers to stimulate HSPD. Three embryos for each age (7-25dpf) were used for these experiments. Movies were recorded using the digital microscope Jiusion 2K HD 2560x1440P USB Digital Microscope using S-EYE[24] or VirtualDub[25] in either .MP4 or .avi format. MP4 movies were converted to .avi using an online converter[26] and decompressed in VirtualDub. For anatomy imaging, Nikon SMZ1500 and Digital Sight DS-U2 camera were used.

To film videos for analysis of contraction profiles of individual chambers, individual 11dpf embryos were placed in agarose moulds. Movies were filmed on Zeiss Primo Star microscope using Labscope 4.3.1 in .avi format then decompressed using VirtualDub.

QUANTIFICATION AND STATISTICAL ANALYSIS

Heart rate, stopping and recovery analysis

ImageJ[27] was used to select the ROI over a single chamber, convert to 8-bit greyscale and export to a TIF image sequence. This image sequence was analysed using a ’Pixel Variance’

algorithm which has been previously applied to the contraction of cardiomyocyte monolayers[28]. In short, the algorithm calculates the variance in the pixel value across frames captured over a known time interval. This time interval corresponds to ~40% of the beat period, i.e. 0.4 seconds for the resting 1 Hz beat rate, equivalent to two frames when sampling at 5.7 fps. Once the variance has been calculated on a pixel-wise basis, the value is averaged across all the pixels in the region of interest (ROI). A trace of this value over time provides a measure of the speed with which the pixel values are changing. For a single beat, there will be two peaks in the speed profile, corresponding to contraction and relaxation. The baseline value of the contraction speed varies between ROIs and samples due to the presence of local features and differences in average intensity. Peaks in the 'contraction speed' traces were then identified using the peak prominence (typically >50% of the peak height) relative to the baseline rather than hardcoding the absolute peak height.

Once the timing of the individual peaks had been identified, the number of peaks within five second windows (starting from time zero) were calculated and divided by two to obtain the number of complete heart beats per five second interval. This 'beat count' data, averaged over a five second window, was attributed to the mid-point of the window (i.e. 2.5, 7.5, 12.5 etc. seconds).

Beat rate was calculated for each embryo, using pre-stimulus beat count data from the first 30 seconds of the movies. Averages were then calculated for each day of development. For each individual, the time taken for beat rate to recover 25% of its pre-stimulus rate was measured. The first point of recovery considered was the 37.5s timepoint as the 32.5s timepoint was not an accurate representation, due to the noise in data following the mechanical stimulation at 30 seconds which often caused the embryos to slightly rotate inside the chorion. For this reason, if heart stops were very short, they were not captured. Averages for time to reach 25% recovery were then taken for each dpf.

To test for significant difference in average beat rates across each age of embryo (7-25dpf) a one-way ANOVA was used. To compare and test for significant difference in beat rates between developing (7-13dpf) and diapause (13-25dpf) embryos, a t-test was used.

Contraction profiles of individual chambers

ImageJ was used to select a ROI over the sinus venosus, atrium and ventricle separately and plot separate z-axis profiles. Data points were extracted and plotted on a single graph for each movie. Drift was removed using a Butterworth high-pass filter, with zero-phase delay.

For three embryos, durations between contractions of individual chambers were measured. For each embryo, four measurements were taken before HSPD and four after. Averages were then taken to compare chamber contraction timings before and after HSPD.

Supplementary figure and video legends

Supplementary figure 1. Time to 25% recovery of pre-stimulation beat rate. For 90-second recordings, stimulation was applied at 30s, inducing HSPD. Beat rate was calculated at 5s intervals, starting from 2.5s. For 7 and 8dpf embryos, time to reach 25% recovery of their beat rate was 0s as they did not show HSPD. Light blue bars show where embryos did not reach 25% recovery within the 90s movie. Orange dotted outlines on these bars show where 3/3 embryos did not reach 25% recovery within the 90s movie, yellow for 2/3 or green for 1/3. Error bars show the minimum and maximum times for each age category to reach 25% recovery, excluding those that did not reach 25% recovery within the 90s movie.

Supplementary video 1. Videos showing other movements during HSPD. Videos are shown for two individual 13dpf embryos. Heart stops were stimulated at 10s. In example 1, eye movement can be seen during HSPD. The heat restarts beating at ~26s. In example 2,

mouth and opercular movement can be seen. The heart restarts beating at ~24s. This shows how HSPD does not cause complete paralysis of the body.

Supplementary video 2. Video highlighting the sensitivity of embryos to physical disturbance. In this video, the embryo is gently nudged using the tip of a tweezer, imitating the movement of a crab claw. This disturbance causes HSPD. This stimulation is administered at ~40s. The beat rate for this embryo was 51bpm. After the stimulation, time to 25% recovery was 87.5s and time to full recovery was 142.5s. This video also highlights the sensitivity of *K. marmoratus* embryos to physical disturbance, causing HSPD.

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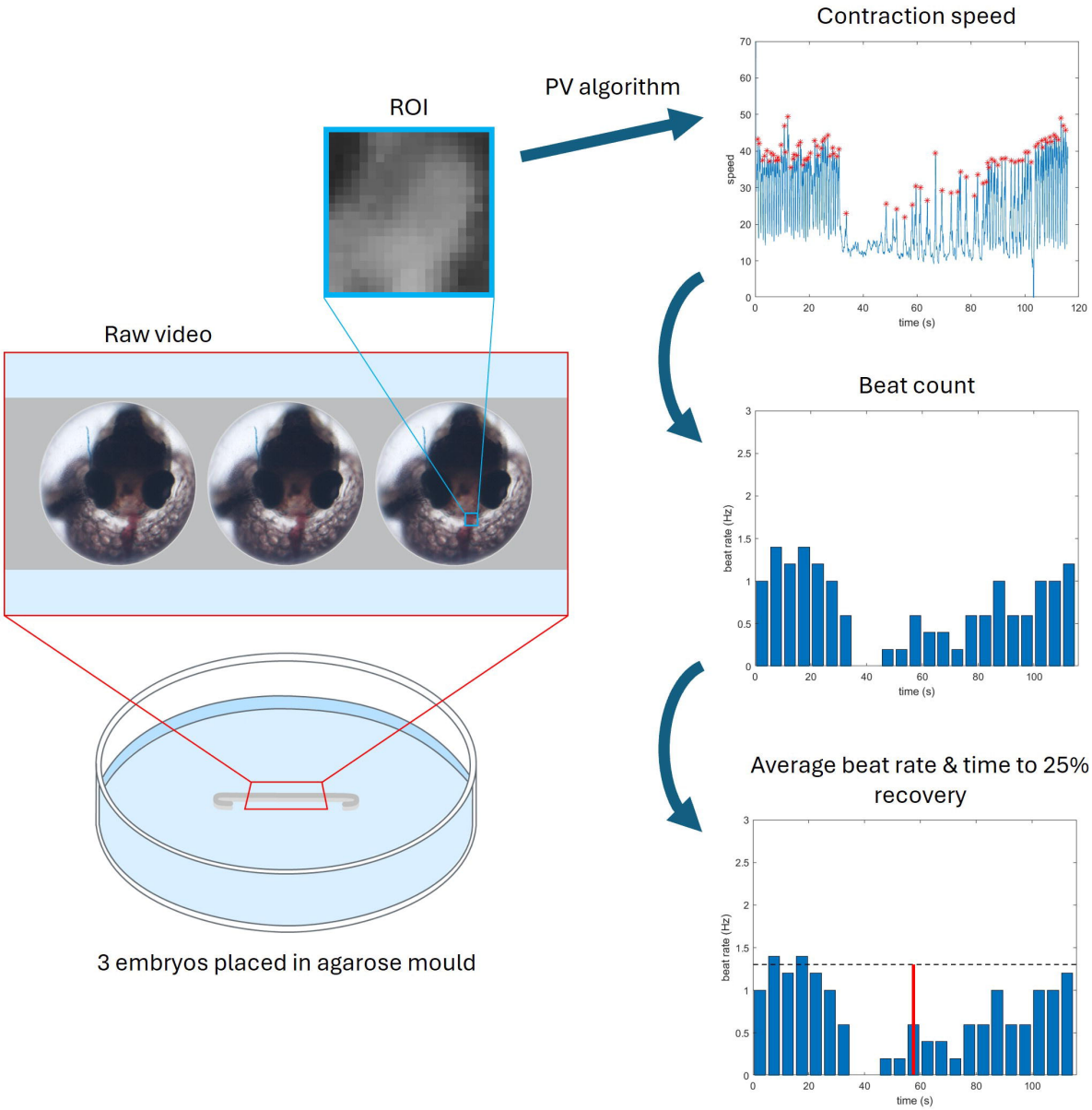
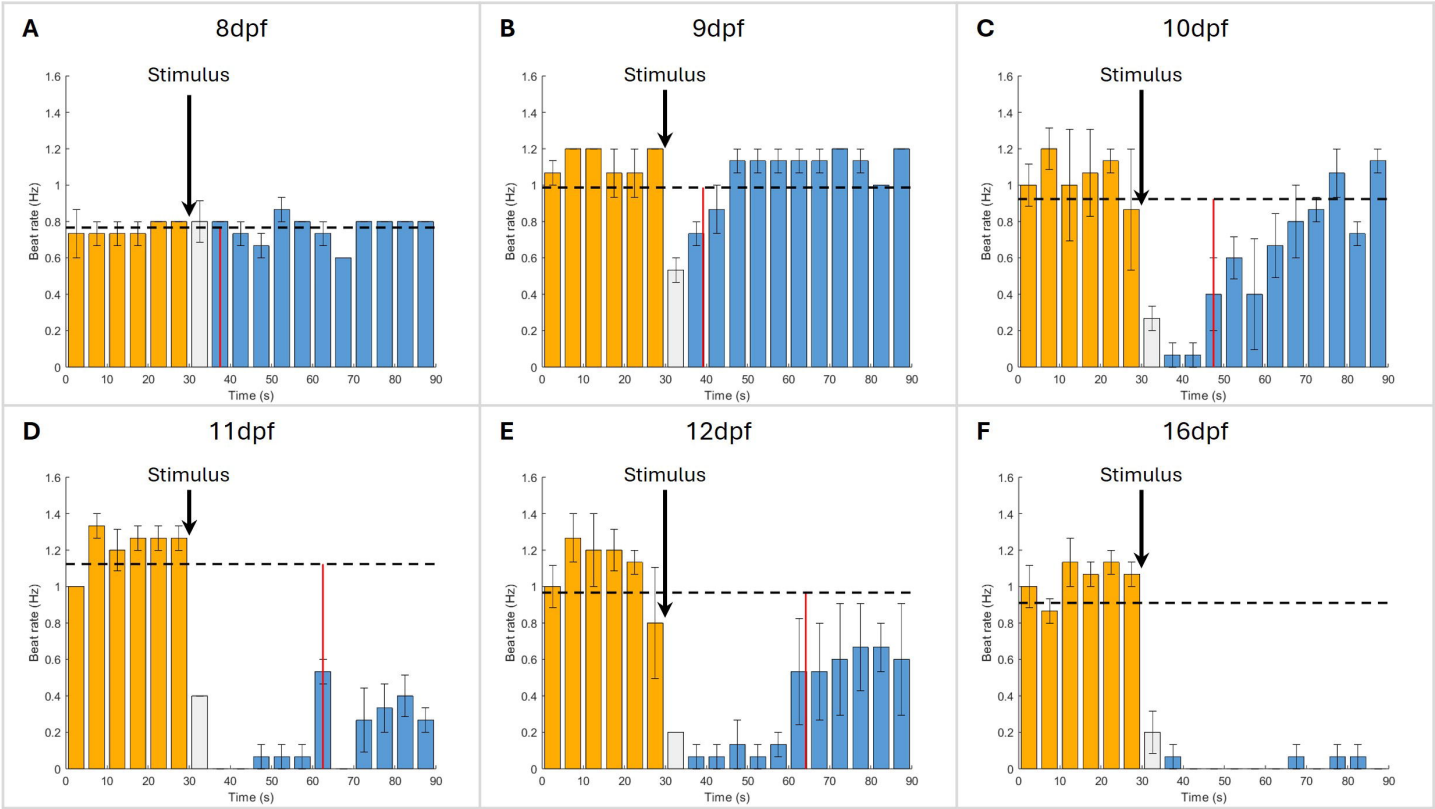
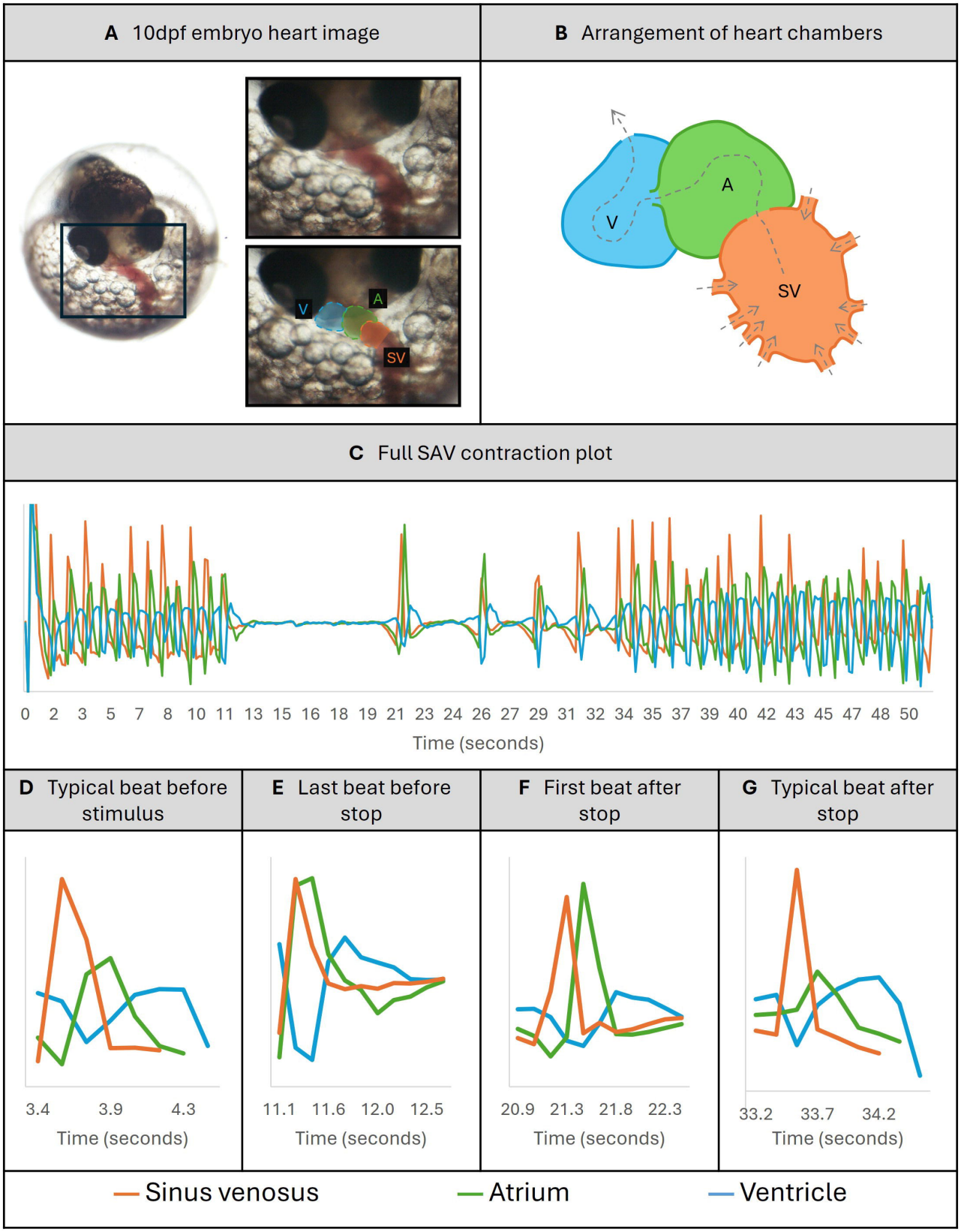
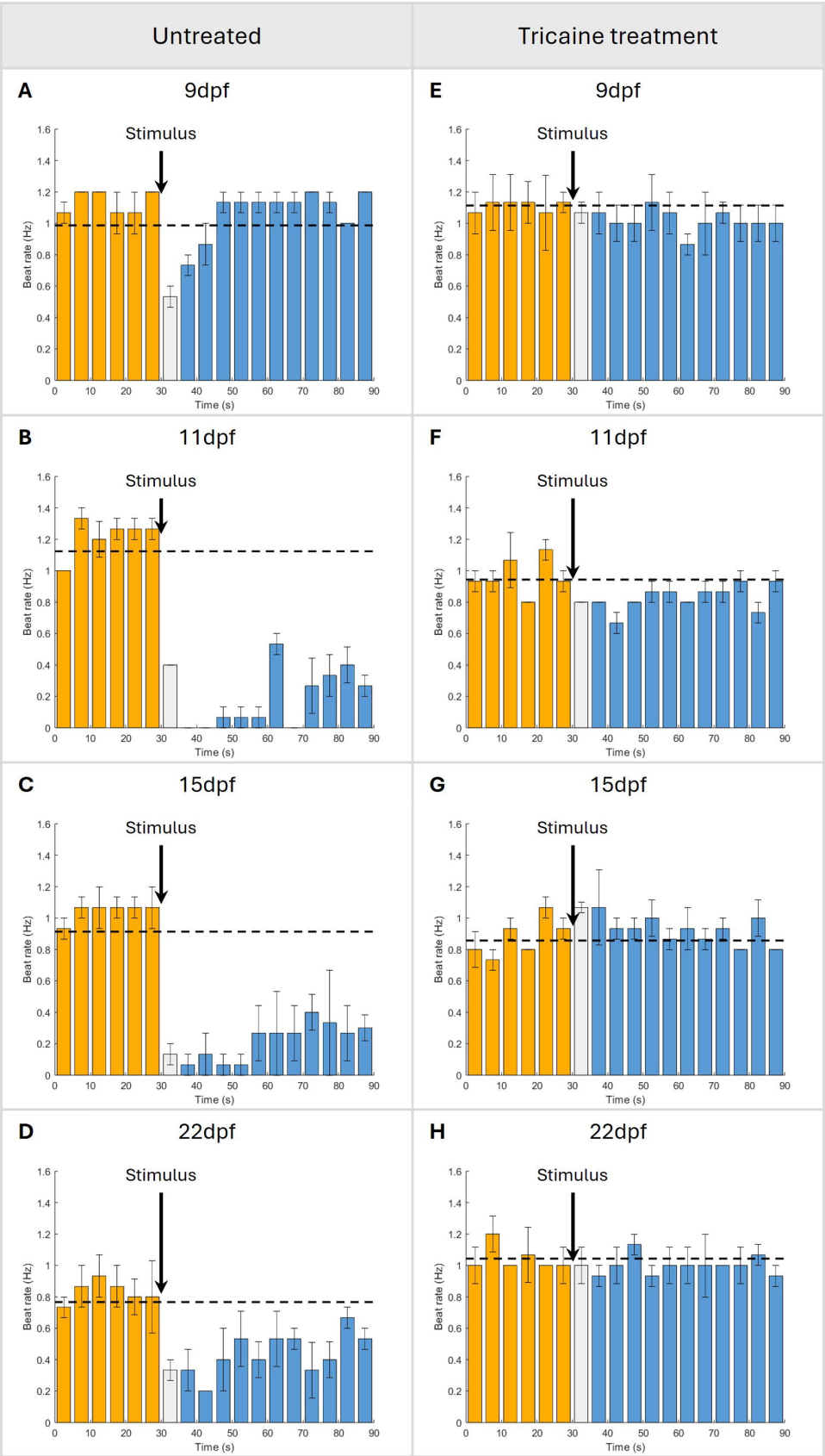

Contraction speed
PV algorithm
ROI
speed
time (s)
Raw video
Beat count
beat rate (Hz)
time (s)
Average beat rate & time to 25% recovery
beat rate (Hz)
time (s)
3 embryos placed in agarose mould


A
8dpf
Stimulus
B
9dpf
Stimulus
C
10dpf
Stimulus
D
11dpf
Stimulus
E
12dpf
Stimulus
F
16dpf
Stimulus
Beat rate (Hz)
Time (s)


A 10dpf embryo heart image
V
A
SV
B Arrangement of heart chambers
V
A
SV
C Full SAV contraction plot
0 2 3 5 7 8 10 11 13 15 16 18 19 21 23 24 26 27 29 31 32 34 35 37 39 40 42 43 45 47 48 50
Time (seconds)
D Typical beat before stimulus
3.4 3.9 4.3
Time (seconds)
E Last beat before stop
11.1 11.6 12.0 12.5
Time (seconds)
F First beat after stop
20.9 21.3 21.8 22.3
Time (seconds)
G Typical beat after stop
33.2 33.7 34.2
Time (seconds)
Sinus venosus
Atrium
Ventricle


Untreated
Tricaine treatment
A
9dpf
E
9dpf
B
11dpf
F
11dpf
C
15dpf
G
15dpf
D
22dpf
H
22dpf
Stimulus
Beat rate (Hz)
Time (s)