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Daily rhythms in heartbeat rate are intrinsic to the zebrafish heart.

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Short title: Control of heartbeat rate by the circadian clock.

Summary

It is a well-established fact that different tissues within the body contain their own circadian clocks or pacemakers, where it is proposed that the clock controls the local, daily cell biology of that organ [1,2]. In mammals, these peripheral clocks work in concert with, and are entrained by rhythmic signals arising from the suprachiasmatic nucleus (SCN) in the hypothalamus of the animal, amongst other systemic cues [2]. In the case of zebrafish, the circadian system appears to be highly decentralized with each tissue not only having an internal circadian clock, but also being directly light entrained [1]. Several years ago, we showed that the zebrafish heart contains its own circadian pacemaker at the gene expression level [1]. This is also the case in mammals, where the circadian clock controls approximately 10% of the cardiac genome [3]. However, heart rate itself is generally thought to be regulated by several well-described autonomic cues, neurotransmitters, and hormones. In this study, we report that, for larval zebrafish hearts, the daily change in heartbeat rate is not only clock-controlled *in vivo*, but that this rhythm also persists *in vitro*, indicating that the cardiac circadian clock itself can directly drive this major physiological oscillation. This result has significant implications for how the circadian clock interacts with the heart pacemaker and regulates aspects of muscle contractility.

Main Text

To examine the heartbeat rate *in vivo* in *Tg((gal4)cmlc2:GFP)* zebrafish larvae, embryos were collected within thirty minutes of laying and placed in an incubator at 28ºC on a 12-h light-12-h dark cycle, provided by LEDs of intensity ~200-400 μW/cm^2. This transgenic line of zebrafish labels the heart with GFP and so makes visualization and surgical excision easier. At 75 hours post fertilization (hpf; Day 3) embryos were removed from the incubator and placed on the temperature-controlled stage of a microscope, where the larval heartbeat was video recorded for >15 sec in 14-27 larvae. 75 hpf corresponds to Zeitgeber Time 3 (ZT3), which represents a time-point 3 h after lights-on. This procedure was repeated every 6 h over the following three days at ZT3, ZT9, ZT15 and ZT21 for Day 3, 4 and 5, using the same pool of embryos. The

results of this counting procedure are shown in Figure 1A, revealing a clear circadian rhythm in heart rate for the *Tg((gal4)cmlc2:GFP)* fish. The heartbeat rate was significantly higher during the day than during the night. Zebrafish embryos typically hatch around Day 3 of development at 28ºC, when they become actively free swimming. Larvae are most active in the day, and so it is perhaps not surprising that higher heartbeat rates correspond to this raised, diurnal activity. Nevertheless, it is quite remarkable that such a robust rhythm in daily heartbeat rate is found so early during *in vivo* heart (and embryo) development.

To prove that this circadian change in heartbeat rate is clock-controlled and not acutely light driven, the experiment was repeated, but with Day 4 and 5 larvae maintained in constant darkness. Heartbeat rate was counted using infrared illumination and an IR-sensitive camera at 850 nm. As the circadian rhythm in the heartbeat rate continued in constant darkness, this suggests that it is clock-controlled and not light driven (Figure 1B). Furthermore, the daily changes seen in the absence of light, address the issue that subtle light-induced temperature rhythms could be driving the LD rhythm. Exposing zebrafish to constant light is known to “stop” the circadian pacemaker and removes any entrainment signal [4,5]. Equally, larvae raised under LL conditions do not experience any rhythmic entraining signals in the environment and, as such, the cardiac circadian pacemaker is likely to be unsynchronized. Under these conditions, the daily rhythm in heartbeat rate was completely absent in developing larvae, as one would predict for a directly clock-regulated process (Figure 1C).

We have previously shown that the zebrafish heart contains its own light responsive circadian pacemaker by studying both rhythmic and light-induced gene expression changes, a result that represented one of the first demonstrations of the existence of peripheral tissue circadian pacemakers [1]. However, relatively little is known about the downstream processes these heart clocks regulate. Other studies have explored molecular, transcriptional targets, but there is still a possibility that the daily rhythm in heartbeat rate is under the control of the endogenous heart circadian clock itself. It is generally assumed that autonomic innervation is responsible

for this physiological daily heart rhythm [3]. To test this idea, we excised larval hearts at ZT2 on Day 3 of development and then placed them into culture for the subsequent two days on a light-dark cycle, with the heartbeat rate counted, as described above. The heartbeat counts are shown in Figure 1D, which reveals the clear and significant diurnal rhythm in heart rate in the cultured tissue. To determine if this rhythm in heartbeat rate is caused by a direct response to the light-dark cycle *in vitro* or is driven by the endogenous circadian clock in the cardiac tissue, we repeated the experiment but transferred the cultured hearts to DD on 4 dpf and continued measurements for two further DD cycles. In this case, to ensure that any rhythm was driven by the endogenous circadian pacemaker and not external influences, the measurements were fully automated in an incubator with time-lapse infra-red microscopy, usingthe same parameters as described above. Consequently, there were no external influences on the cultured hearts once placed into DD. The hearts are clearly rhythmic in DD, with a significant oscillation in heartbeat rate on the second cycle (Figure 1E). These results clearly demonstrate that the circadian clock contained within the cardiac tissue can drive a daily rhythm in heartbeat rate, which is not controlled by the environmental light-dark cycle. Such a result raises several intriguing questions that will require further study, such as: How does the circadian clock interact with the heartbeat pacemaker in cardiomyocytes to cause this daily change? It also raises the likelihood that the circadian pacemaker is interacting at the cellular level with Ca^{2+} oscillations in the cardiomyocytes to generate this daily rhythm, but numerous other possibilities exist. The actual number of heart beats is much lower *in vitro* than *in vivo*; this supports the idea, perhaps not surprisingly, that systemic cues, both neuronal and hormonal, are regulating heart function within the whole animal. Of course, it is also possible that the culture environment itself is simply not optimal for heart function. What is the comparative situation with mammalian cardiac tissue? Cultured mouse cardiomyocytes and ventricular explants have been shown to contain a cell autonomous circadian pacemaker, similar to zebrafish, but without the direct light-sensitivity [6]. Moreover, a direct coupling of the clock to the circadian heartbeat rate was not detected in these studies. Transcriptomic studies on mammalian cardiac tissue reveal a wide range of daily molecular changes *in vivo*

and clock-disruption can reduce the expression of genes, such as the *α-adrenoceptor*. This will clearly alter the heart's ability to respond to systemic regulatory signals [3,7,8]. In addition, there are reported daily changes in Ca^{2+} channel expression [9], which will have a clear impact on cardiac function, and this raises the distinct possibility that the circadian pacemaker might also couple directly to regulate muscle contraction in mammals.

To conclude, in this study, we show that the circadian clock can control the daily heartbeat rate from the earliest stages of embryo development. Furthermore, we show that the endogenous cardiac circadian pacemaker directly couples to the heartbeat rate and drives a daily rhythm in this process. These results lay the foundations for a wide range of future studies relating to how the circadian clock within cardiomyocytes regulates downstream cardiac muscle contractility and interacts with the intrinsic heartbeat pacemaker.

Acknowledgments

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Figure 1. The change in heartbeat rate of *Tg((gal4)cmlc2:GFP)* larvae from 3 dpf to 5 dpf in different lighting conditions. Larvae were maintained in: (A) alternating light and dark (LD) conditions continually; (B) LD until 85 hpf, after which they were placed in constant dark (DD); or (C) constant light (LL) starting as soon as the eggs were fertilized. In each case, the heartbeat rates were recorded every 6 h from 3 dpf until the end of day 4. In these graphs, the yellow segments indicate the light phases; the dark grey segments indicate the dark phases, and the light grey segments indicate the phases when the entrained (now free running) zebrafish circadian clock would have experienced light. In (A-C), the peak and trough values at ZT3 and ZT15 for each fish line were compared using the Student's t-test. The asterisks indicate significant differences at $P<0.05$ (*), $P<0.005$ (**) or $P<0.0005$ (***) . In (A), (B) and (C), n = 20, 22 and 18 larvae, respectively, per experiment for 3 experiments conducted on different days. ZT is zeitgeber time; CT is circadian time. In (D), excised hearts were maintained in culture on a light-dark cycle and heartbeat rate was counted for the following two-days (n=25). In (E), excised hearts were maintained on an LD cycle for one day before entering constant dark (DD) free-running conditions for a subsequent cycle (n=12). The daily rhythm in heartbeat rate is clearly maintained under constant conditions *in vitro*, demonstrating direct circadian clock-control of this physiological process. See Supplementary Figure 1 for additional free-running data in constant darkness.

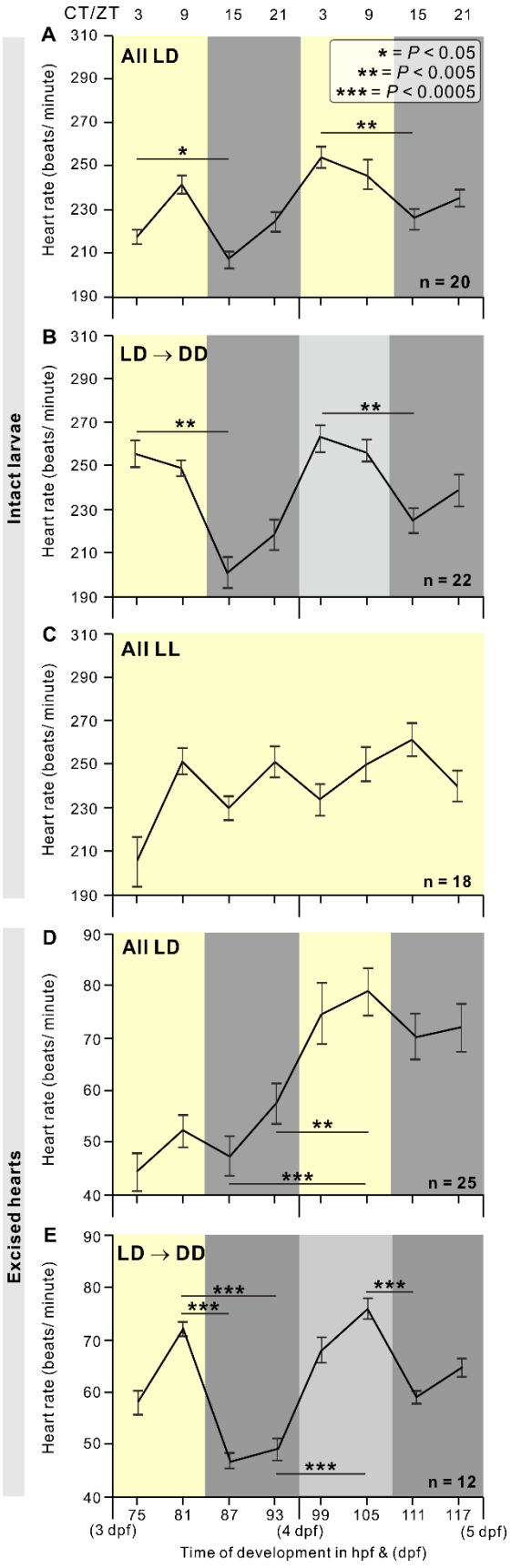
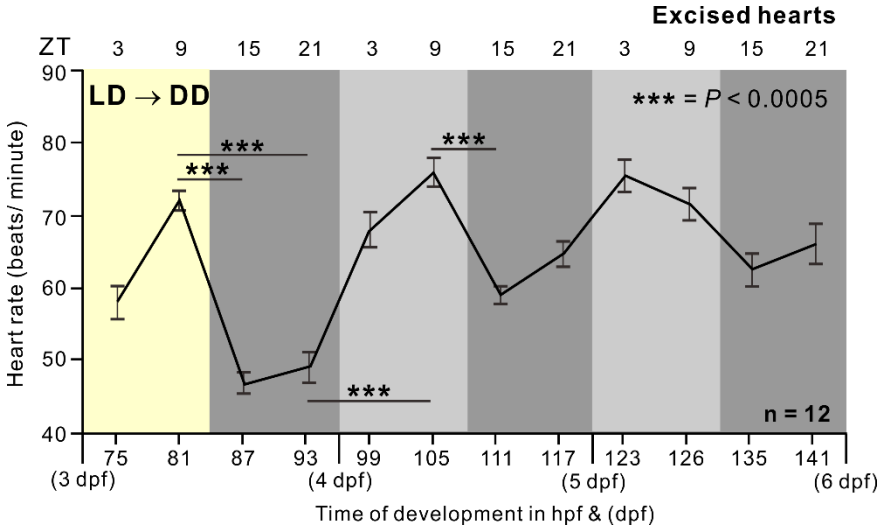

CT/ZT 3 9 15 21 3 9 15 21
A
All LD
* = P < 0.05
** = P < 0.005
*** = P < 0.0005
n = 20
B
LD → DD
n = 22
C
All LL
n = 18
D
All LD
n = 25
E
LD → DD
n = 12
Intact larvae
Excised hearts
Heart rate (beats/ minute)
75 81 87 93 99 105 111 117
(3 dpf) (4 dpf) (5 dpf)
Time of development in hpf & (dpf)

Fig. 1

Supplementary Information

Supplementary Figure 1.



The Supplementary Figure shows the same data for excised hearts, as shown in Figure 1 of the main manuscript, but with an additional day (Day 5-6) in constant darkness added. This additional data clearly strengthens the argument that the rhythm in heartbeat rate continues to free-run under constant dark conditions, proving direct circadian clock-control of this physiological process.