Autonomic Control of Heart Rate Variability in Emu (*Dromaius novaehollandiae*) Hatchlings

Rajesh Motiwala and Edward M. Dzialowski*

Department of Biological Sciences
P.O. Box 305220
University of North Texas
Denton, TX 76203, USA

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*Corresponding Author: Edward M. Dzialowski
edzial@unt.edu

Department of Biological Sciences
University of North Texas
P.O. Box 305220
Denton, TX 76203, USA

Phone: 940-565-3631
Fax: 940-565-3821
ABSTRACT

In mammals, heart rate variability (HRV) is a consequence of variable input from the sympathetic and parasympathetic branches of the autonomic nervous system. To date, the origin of heart rate variability in lower vertebrates has received scant attention. We examined the role of the autonomic nervous system in regulating heart rate and HRV in day-old emu hatchlings maintained at their incubation temperature of 36.5°C. The role of the autonomic nervous system in controlling emu HRV was examined by blocking the action of the cholinergic and adrenergic pathways by administration of atropine and propranolol. The mean heart rate of day-old hatchlings exhibited a significant cholinergic tone of 60 bpm (SD 22) and β-adrenergic tone of 28 bpm (SD 17). Initially, all embryos exhibited Type I HRV with a peak frequency of 0.22 Hz (SD 0.01), associated with respiratory sinus arrhythmia. After cholinergic blockade, the spectral power of instantaneous heart rate decreased significantly in the high (0.1 to 0.3 Hz), low (0.01 to 0.1 Hz), and total frequency components. This was associated with a loss of the power spectrum peak corresponding to a respiratory sinus arrhythmia. While β-adrenergic blockade produced a decrease in mean heart rate, it had no effect on respiratory sinus arrhythmia or the high, low, and total frequency component of HRV. We conclude that while both the sympathetic and parasympathetic system exert a tonic influence on heart rate, the majority of the high and low frequency variability observed in instantaneous heart rate is mediated by the parasympathetic system in the emu hatchling.

Key words: respiratory sinus arrhythmia, sympathetic, parasympathetic, heart rate variability, bird
INTRODUCTION

Variability in beat-to-beat heart rate is a common feature of the vertebrate cardiovascular system (3, 5, 9, 27, 28). To date, the majority of studies have examined the source and consequences of heart rate variability in the mammalian system (2, 24). The avian egg has been used for years as a model for developmental studies of the cardiovascular system, however very little is known about the origin of heart rate variability in developing birds. Recently, studies have begun to focus on the heart rate variability in developing chicken and emu (14, 17-20, 25, 28).

A number of basic heart rate variability patterns have been categorized in the avian system based on the frequencies associated with major peaks in the heart rate power spectrum (13, 18, 19). The emu hatchling exhibits heart rate variability in both high frequency (HF; 0.1 to 0.4 Hz) and low frequency (LF; 0.01 to 0.1 Hz) ranges (13, 19). Hatchlings and pipped embryos that exhibit a Type I heart rate variability have a wide baseline instantaneous heart rate that oscillates with a regular high frequency. In the emu hatchling, Type I heart rate variability occurs with a frequency ranging from 0.2 to 0.4 Hz (19). In the newly hatched chicken, the frequency of Type I heart rate variability and the hatchlings’ respiration rate are similar (17). Thus, Type I heart rate variability is thought to be a respiratory sinus arrhythmia mediated by the cardiac vagal nerve of the parasympathetic system (6, 14, 19, 28). Type II heart rate variability is associated with LF oscillations and occurs with a main peak frequency of 0.07 Hz in the emu (19, 25). In the chicken hatchling, these LF peaks appear to be associated with thermoregulation (14). They occur with much less frequency than Type I oscillations in
the emu (25). These patterns in heart rate variability in the avian system fall within the same frequencies as mammals (26).

In the mammalian system, HF and LF heart rate variability are regulated by inputs from the sympathetic and parasympathetic system (1, 22, 24). Heart rate variability occurring in the HF range is mediated by the parasympathetic system (2), while a combination of inputs from both systems is thought to control LF heart rate variability. In the emu and chicken a functioning cholinergic and β-adrenergic tone on heart rate is present beginning at 70% of incubation, suggesting that both branches of the autonomic nervous system are functional upon hatching (6-8). While a functional autonomic tone exists in the preterm embryo, the role of the autonomic nervous system in the control of avian heart rate variability remains unclear. In the present study, we investigated the influence of the parasympathetic and sympathetic systems on mean heart rate, Type I heart rate variability, and HF and LF heart rate variability in emu (Dromaius novaehollandiae) hatchlings.

MATERIALS AND METHODS

Emu (Dromaius novaehollandiae) eggs were obtained from the M. Pearson Ranch in Pilot Point, TX, brought to the University of North Texas, and incubated in a Hatchrite forced draft incubator maintained at 36.5°C with a relative humidity of ~30%. Eggs were automatically turned every 4 hours. On day 49 of incubation, eggs were transferred to a hatching incubator with a temperature of 36.5°C and a relative humidity of ~35% and allowed to hatch. All experiments were carried out on hatchlings within 24
hours of hatching. The University of North Texas Institutional Animal Care and Use Committee approved the experimental protocol.

Instantaneous heart rate was determined from an electrocardiogram. To facilitate contact between the electrocardiogram electrodes and the skin, a hatchling's feathers were trimmed laterally from under each wing and the abdomen. Three electrodes were attached to the hatchling, one under each wing and one on the abdomen with gauze tape and electrocardiogram paste to create better contact with the skin. The hatchlings were then placed into a holding chamber in a temperature controlled incubator maintained at 36.5°C for the duration of the testing. The electrocardiogram signal was preconditioned with a Grass P55 A.C. pre-amplifier and was measured using an ADInstruments 8SP Powerlab and Chart 5.02 software with a sampling frequency of 100 Hz (n=5) or 2000 Hz (n=11).

The role of the autonomic nervous system in regulating mean heart rate and heart rate variability in emu hatchlings was studied by administering antagonists of the sympathetic and parasympathetic nervous systems. Atropine (1 mg/kg) was used to block the parasympathetic cholinergic receptors. Propranolol (3 mg/kg) was used to block the β-adrenergic receptors of the sympathetic system. Prior to the injection of either drug, the instantaneous heart rate was recorded for approximately one hour to establish a baseline instantaneous heart rate. After one hour, 1 mg/kg atropine was injected intraperitoneally into the hatchling. Care was taken during injections to minimize any disturbance or stress on the animal. The instantaneous heart rate measurement was continued for approximately 20 minutes following the initial cholinergic blockade. This was followed by an intraperitoneal injection of 3 mg/kg
propranolol and an additional twenty minutes of instantaneous heart rate recordings. This procedure was conducted on seven hatchlings. In eight additional hatchlings, the above procedure was carried out but the order of drug administration was reversed.

**Statistical and power spectrum analysis**

Instantaneous heart rate was calculated as the beat-to-beat interval of adjacent R waves and expressed in units of beats min$^{-1}$ (bpm). Changes in heart rate variability were analyzed with the ADInstruments HRV module for Chart 5.02. The heart rate variability module provided R-R intervals from 5 min selections from the raw electrocardiogram data. Power spectra were calculated by Fast Fourier transformation with 256 data points using a Welch windowing function with one-half overlap. The HRV module resampled the R-R intervals to obtain equidistant points for FFT. To determine the presence of Type I heart rate variability, we examined each power spectra for any prominent peaks in the expected range of 0.18 to 0.4 Hz (Tamura et al., 2003; Moriya et al. 2002) and noted the frequency at which the peak was strongest. We calculated the power of the total heart rate spectrum, the low frequency component (0.01 to 0.1 Hz), and the high frequency component (0.1 to 0.3 Hz) by integrating the power spectra across each frequency range. The powers were used to determine the link between the sympathetic and parasympathetic systems and HF and LF heart rate variability.

The influence of the parasympathetic and sympathetic systems on mean instantaneous heart rate and heart rate variability were analyzed with one-way repeated measures ANOVA. Because LF power, HF power, and total power had unequal variances, statistics were run on the log-transformed data. The Holm-Sidak post hoc test was used to examine differences between treatments and control values when the
repeated measures ANOVA was found to be significant. All statistical tests were carried out using Sigma Stat 3.0.1 (SPSS). The level of significance for all tests was P < 0.05. All values are reported as the mean (standard deviation).

RESULTS

Cholinergic and Adrenergic Tone

The administration of autonomic blocking agents had a significant effect on hatchling mean instantaneous heart rate (P<0.001). Figure 1 summarizes the changes in mean heart rate after the administration of both sequences of atropine and propranolol. After cholinergic blockade, mean heart rate increased significantly by 60 bpm (SD 22) (Fig. 1A). β-adrenergic blockade by propranolol produced a significant decrease in mean heart rate of 35 bpm (SD 22) below the level established with cholinergic blockade, but still significantly higher than the baseline instantaneous heart rate. When the β-adrenergic system was blocked first, mean heart rate significantly decreased by 28 bpm (SD 17) (Fig. 1B). This was followed by blockade of the parasympathetic system, producing a significant increase in mean heart rate of 56 bpm (SD 21).

Heart Rate Variability

A representative trace of instantaneous heart rate and the corresponding power spectra for a day old hatchling under no blockade (control), cholinergic blockade, and cholinergic plus β-adrenergic blockade is shown in Fig. 2. Initially, hatchling instantaneous heart rate exhibited a wide oscillation of 18 bpm around the mean baseline heart rate (Fig. 2A). The power spectrum for this animal had a prominent peak at 0.26 Hz prior to cholinergic blockade (Fig. 2B). In all hatchlings receiving atropine
first, a peak was present in the control power spectra with a mean of 0.22 Hz (SD 0.02; n=6; Table 1). Following cholinergic blockade, the peak in the power spectrum between 0.2 and 0.3 Hz was no longer present in this or any of the other animals (Fig. 1D; Table 1). The loss of the Type I HF variability can also be seen in Fig. 1C as the instantaneous heart rate fluctuated by less than 1 bpm around the mean heart rate with cholinergic blockade. The addition of β-adrenergic blockade did not bring about any further significant changes in the heart rate variability or its power spectra (Fig 1E and 1F).

A representative trace of instantaneous heart rate and the corresponding power spectra of a day old hatchling under no blockade, β-adrenergic blockade, and β-adrenergic plus cholinergic blockade condition is shown in Fig. 3. This animal exhibited a fluctuation of 12 bpm around the mean heart rate (Fig 3A). As with the animals receiving cholinergic blockade first, a peak in the HF range of the power spectrum (0.23 Hz) appeared in the control condition for this and all other animals receiving β-adrenergic blockade first (Fig 2B; Table 1). Upon β-adrenergic blockade, instantaneous heart rate continued to vary around the baseline by approximately 12 bpm (Fig 3C). The peak in the power spectrum corresponding to the Type I heart rate variability remained after β-adrenergic blockade in this animal and every other animal (Fig. 3C; Table 1). Cholinergic blockade with atropine resulted in instantaneous heart rate fluctuations of less than 1 bpm around the mean (Fig 3E) and disappearance of the peaks in the HF range of the power spectrum of all animals (Fig 3F; Table 1).

Table 1 shows the effects of autonomic blocking agents on the area of the heart rate power spectra in the HF (0.1-0.3 Hz), LF (0.01-01 Hz), and total frequency ranges.
When the cholinergic blockade occurred first, it produced a significant decline compared to the control in the power for all frequency ranges examined (P<0.001; Table 1). The subsequent addition of propranolol caused the HF and LF powers to remain suppressed with no further decrease from the established cholinergic blockade values.

A one-way analysis of variance revealed a significant change in the high, low, and total frequency powers in the hatchlings subjected to β-adrenergic blockade followed by cholinergic blockade (P<0.001). Initial β-adrenergic blockage with propranolol had no effect the HF power, LF power, or total power (Table 1). However, a significant decrease occurred after parasympathetic blockage with atropine, where the high, low, and total powers all decreased significantly from both the control and the propranolol injection values.

**DISCUSSION**

**Cholinergic and Adrenergic Heart Rate Tone**

The changes in mean heart rate that occurred following the administration of atropine and propranolol support the presence of an active cholinergic and β-adrenergic tone regulating mean heart rate in the emu hatchling. The magnitude of the hatchling cholinergic and β-adrenergic tone is similar to the tone observed in the embryonic emu (7). In the embryonic emu, a β-adrenergic and cholinergic tone on heart rate was observed as early as day 35 of a 50-day incubation period (70% of the total incubation period). Both autonomic tones were initially weak at 70% of development and increased during the later stages of incubation (7). There was a slight increase in the cholinergic mediated tone in emu hatchlings compared with that of the emu embryo at
90% of the incubation period (+10 bpm). Additionally, the β-adrenergic receptor mediated tone was slightly lower in the emu hatchlings compared with emu embryos at 90% of incubation (-14 bpm). After blockade of the cholinergic and β-adrenergic receptors, the emu hatchling exhibited a slightly higher intrinsic heart rate than the developing embryo.

The fetal and neonatal sheep have a similar pattern of development in the cholinergic receptor-mediated heart rate tone (30, 32). A weak cholinergic tone occurred as early as 60% of gestation in the fetal sheep and increased through development (30). Upon birth, the cholinergic tone on heart rate continued to increase in the neonatal sheep (30, 32). The development of a β-adrenergic tone on heart rate in the developing sheep is not as clear. One study observed a decrease in β-adrenergic receptor mediated tone with the transition from the fetal to neonatal stage (32), while another study showed that β-adrenergic receptor mediated tone increased upon birth (30). The developmental pattern of cholinergic tone and β-adrenergic tone on heart rate are similar in the emu and the sheep.

Resting heart rate in the emu hatchling is a combination of cholinergic and β-adrenergic tonic inputs. Interestingly, the resting heart rate in white leghorn chicken embryos was shown to rely more on sympathetic tone rather than parasympathetic tone prior to hatching, which suggests development of parasympathetic tone is slowed and must occur post-pipping in this breed of chicken (8). In contrast, Chiba et al. (6) found that on the 18th day of incubation in a different breed of chicken, the broiler chicken, embryos exhibit a parasympathetic tone. While conflicting data have been reported for the developing chicken, the emu develops a clear cholinergic and β-adrenergic heart
rate tone during the last 1/3 of *in ovo* development and this is maintained upon hatching.

The adult emu has a heart rate that is 1/3 the heart rate of 0 to 7 day old emu hatchlings (10, 13, 19). It is possible that the cholinergic and β-adrenergic tone continue to mature as the animal grows to the adult stage. However, decreases in resting heart rate of the neonatal sheep during the first 8 weeks was shown to be due to changes in the intrinsic heart rate and not changes in parasympathetic or sympathetic tone (32, 33). Further study is needed in birds to determine how regulation of heart rate changes as the hatchling develops into an adult.

**Heart rate variability**

The majority of the variability observed in emu hatchling heart rate is mediated by the parasympathetic branch of the autonomic nervous system (Table 1). Blocking the cholinergic receptors with atropine abolished the power in the HF, LF, and total frequency ranges in the emu hatchlings. In contrast, β-adrenergic blockade while producing a decrease in instantaneous heart rate, had no effect on heart rate variability at any of the frequencies examined.

All hatchlings exhibited a clear HF peak in the heart rate power spectrum corresponding to Type I heart rate variability with a mean frequency of 0.22 Hz. A similar HF peak has been previously observed in both chicken and emu hatchlings and is thought to be a respiratory sinus arrhythmia mediated by the vagus nerve (14, 19, 20). Data supporting a link between avian heart rate variability and respiration comes from chicken hatchlings where the peak frequency of the Type I heart rate variability is the same as the respiratory frequency (4, 17). In the day old emu hatchling, the HF
spectral peak occurs at 0.22 Hz and was completely abolished by cholinergic blockade in this study (Table 1). Sympathetic blockade prior to cholinergic blockade produced a slight increase in the frequency at which the peak in the Type I heart rate variability occurred, but there was no change in the power of the peak (Table 1). These findings suggest that the Type I heart rate variability in the emu is a respiratory sinus arrhythmia that is mediated by the parasympathetic system. This origin of a parasympathetic driven respiratory sinus arrhythmia is in agreement with the mammalian system (1, 2, 11, 22, 31).

In addition to the specific respiratory sinus arrhythmia in the emu hatchling, there was a large amount of non-frequency specific spectral power in the HF range. As with the respiratory sinus arrhythmia, parasympathetic inputs accounted for the majority of the HF variability with cholinergic blockade producing a 98% decrease in the HF spectral power. In contrast, administration of propranolol produced only a slight, non-significant decrease in the HF spectral power when administered first. In the hatchling emu, the sympathetic system has very little influence on HF heart rate variability. A similar dominant role of the parasympathetic system in HF heart rate variability has been observed in the developing chicken embryo (6). Administration of atropine to 18-day-old chicken embryos significantly diminished the HF spectral power to near 0. It appears that in the developing avian embryo and hatchling, HF heart rate variability is dominated by the parasympathetic system.

Our findings that avian HF heart rate variability is under parasympathetic control are in line with those of a number of mammal species, where the HF variability is solely mediated by parasympathetic inputs (12, 31). In 6 month old mice, cholinergic blockade
significantly reduced the HF spectral power between 0.1 and 1 Hz (12). β-adrenergic blockade produced a decrease in mean heart rate, but no change in the HF spectral power in the mouse (12). Thus, the sympathetic system of the mouse produces a β-adrenergic tone but has no contribution toward the HF heart rate variability, just as in the emu hatchling. Similar changes in HF spectral power in response to atropine and propranolol have been observed in humans, dogs, and rats (11, 16, 21).

Comparisons of mammals at the same stage of development as the emu hatchling suggest that the β-adrenergic and cholinergic systems make different contributions to HF heart rate variability in the developing mammal and the emu hatchling. The parasympathetic system plays a large role in the HF variability in the fetal and neonatal lamb. Kimura et al. (15) found that when they administered atropine to fetal lambs, the HF variability was significantly reduced, suggesting a link between the parasympathetic system and HF variability in heart rate. This held true for neonatal sheep, where the use of atropine as a vagal blocking agent led to a large decrease in HF power (23). Unlike the emu, blockade of the β-adrenergic receptors in both fetal and neonatal lambs produces marked decreases in the HF power (15, 23). In neonatal sheep, β-adrenergic blockade produced a significant reduction in the spectral power in the HF range, and this response was most pronounced in lambs younger than 30 days (23). Similar responses of the spectral power of HF heart rate variability were found under β-adrenergic blockade in fetal lambs (15). In both the fetal and neonatal lamb, it appears that β-adrenergic blockade decreases the HF spectral power, but does not change the respiratory sinus arrhythmia. Unlike the emu hatchling, cholinergic and β-
adrenergic pathways mediate the HF heart rate variability in the fetal and neonatal lamb.

As with the HF variability, the parasympathetic division of the autonomic nervous system has a stronger influence on LF heart rate variability of the emu hatchling than the sympathetic division. Cholinergic blockade reduced the LF spectral power and accounted for up to 98% of the LF power at this stage of development in the emu (Table 1). Administration of propranolol produced a slight increase in the LF power, but this increase was insignificant. Cholinergic inputs mediate both the HF and the LF heart rate variability in the hatchling emu. In chicken embryos, the administration of atropine caused a marked decrease in spectral power in the LF range, but did not abolish all of the LF variability (6). Unlike the emu hatchling, there was still a high level of spectral power present in the LF range after cholinergic blockade in the chicken embryo, which suggests that the sympathetic branch may play a larger role in regulating LF variability in the chicken. This potential presence of the sympathetic mediated LF variability in the chicken embryo may reflect either differences in sympathetic maturation between the two species or differences in the stage of development at which the measurements were made (i.e. in ovo vs. ex ovo). Similar to the chicken embryo, LF heart rate variability in the fetal and neonatal lamb is controlled by both sympathetic and parasympathetic inputs (15, 23). β-adrenergic inputs accounted for 78% of the LF power in one group of lambs and the parasympathetic system accounted for 45% of the LF power in another group of animals (15).

The lack of a sympathetic component to emu heart rate variability may have been due to a number of factors. The propranolol used in this study was able to block
the presence of a β-adrenergic tone on heart rate, but no changes in heart rate variability occurred. Thus, the propranolol was functioning properly to block the β-adrenergic receptors of the sympathetic system. It was expected that the sympathetic system would play a large role in the LF component of heart rate variability. In the emu hatchling, Type II heart rate variability associated with LF variability has been observed during long-term heart rate measurements (19). While the animals in this study did not exhibit a spectral peak corresponding to Type II LF heart rate variability, chicken hatchlings have been shown to exhibit a peak in heart rate variability in this LF range under thermal stress (14, 29). The presence of this LF peak appears to be associated with vasomotor control during thermoregulation in a cool environment. Exposure to high air temperatures in the chicken hatchling resulted in the elimination of the LF Type II heart rate variability (14). However, exposure to lower air temperatures rarely produced Type II heart rate variability in the emu (25). All of our hatchlings were maintained at their incubation temperature (36.5 °C) prior to and during the measurements. It is possible that under thermal conditions that are not stressful, Type II variability and sympathetic modulation of LF variability may not occur. Additionally, it has been shown that posture influences the extent of the sympathetic influence on LF heart rate variability (21). In adult humans, standing produced LF heart rate variation that was jointly mediated by both the sympathetic and parasympathetic systems but, when in a supine position, the parasympathetic system dominated LF heart rate variability. The LF heart rate variability may be a baroreceptor response to blood pressure fluctuations. During this study, the emu hatchlings tended to be in a quiet head down sitting position. The posture of the emu hatchling during our measurements may suppress the
baroreceptor response and sympathetic inputs as in the supine human. At this stage of
development, emu hatchlings possess an active baroreflex in response to hypertensive
and hypotensive mean arterial pressure (Unpublished data, E. Dzialowski). Under
certain conditions, not tested here, it is possible that the sympathetic system of an emu
hatchling may contribute to the LF heart rate variability. Finally, the lack of a
sympathetic component to LF heart rate variability may be due the fact that sympathetic
control of LF variability may not be fully developed in the emu upon hatching.

**Perspectives**

The two branches of the autonomic nervous system differentially mediate emu
hatchling heart rate and heart rate variability. As in the developing embryo, emu
hatchlings exhibited both a cholinergic and adrenergic tone on heart rate. Heart rate
variability at both the high and low frequencies is dominated by parasympathetic inputs.
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15. Kimura Y, Okamura K, Watanabe T, Murotsuki J, Suzuki T, Yano M, and Yajima A. Power spectral analysis for autonomic influences in heart rate and


Values are mean (SD). All animals prior to the injection of atropine. Regardless of drug sequence, exhibited a peak values with in are significantly different from injection 1 at P<0.05. Values associated with Type I heart rate variability Values with in are significantly different from the control at P<0.05.

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<td>776.1 (SD 667.6)</td>
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<tr>
<td>LF Power</td>
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<td>7.52 (SD 24.7)</td>
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<tr>
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<td>9.34 (SD 54.7)</td>
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Table 1. Frequency of Type I Heart Rate Variability (HRV), High Frequency (HF: 0.1 to 0.3 Hz), Low Frequency (LF: 0.04 to 0.1 Hz), and Total Power of HRV, and Total Power of LF and HF Power of ECG during administration of atropine and antagonists.
FIGURE LEGENDS

Figure 1. Mean instantaneous heart rate (bpm) of day old emu hatchlings before and after administration of autonomic nervous system antagonists. Values are mean (SD). Values with an * indicate they are significantly different from the control value at P<0.05. Values with an \( \phi \) indicate they are significantly different from injection 1 at P<0.05.

Figure 2. Instantaneous heart rate and the corresponding power spectra before and after the administration of atropine. (A) Instantaneous heart rate and the corresponding power spectra (B) of a day old emu hatchling prior to injection of atropine. The power spectrum shows a prominent peak between 0.2 and 0.3 Hz corresponding to respiratory sinus arrhythmia. (C) Instantaneous heart rate and the corresponding power spectra (D) following cholinergic blockade. (E) Instantaneous heart rate and the corresponding power spectra (F) following cholinergic + \( \beta \)-adrenergic blockade.

Figure 3. Instantaneous heart rate and the corresponding power spectra before and after the administration of propranolol. (A) Instantaneous heart rate and the corresponding power spectra (B) of a day old emu hatchling prior to injection of propranolol. (C) Instantaneous heart rate and the corresponding power spectra (D) following \( \beta \)-adrenergic blockade. (E) Instantaneous heart rate and the corresponding power spectra (F) following \( \beta \)-adrenergic + cholinergic blockade.
Fig. 2

(A) Instantaneous Heart Rate (bpm) vs. Time (min)
(B) Power vs. Frequency (Hz)
(C) Instantaneous Heart Rate (bpm) vs. Time (min)
(D) Power vs. Frequency (Hz)
(E) Instantaneous Heart Rate (bpm) vs. Time (min)
(F) Power vs. Frequency (Hz)