Having the heart to fly. Neontological insights on cardiac performance in the evolution of avian flight

Jordi Altimiras[1], Isa Lindgren[1], Lina María Giraldo-Deck[2], Alberto Matthei[3] and Álvaro Garitano-Zavala[2]

[1] AVIAN Behavioral Genomics and Physiology, Department of Physics, Chemistry and Biology, Linköping University, Sweden

[2] Instituto de Ecología, Universidad Mayor de San Andrés, La Paz, Bolivia

[3] Tinamou Chile S.L, Los Angeles, Chile

Corresponding authors:

Jordi Altimiras

IFM Biology, Linköping University

SE-58183 Linköping, Sweden

Email: jordi.altimiras@liu.se

Phone +46 13 285824

Álvaro Garitano-Zavala

Email: agaritanozavala@umsa.bo

Running head: Heart size in tinamous limits aerobic performance

ABSTRACT

Interpretations on the origin of sustained flight in birds are mainly driven by biomechanical studies from fossil evidence and have largely neglected how the metabolic requirements of flight would have been supported. We studied tinamous, a taxon of flying palaeognathous birds with the smallest heart among birds to evaluate the hypothesis that heart size restricts aerobic performance and hinders sustained flapping flight, findings that could provide new arguments to the avian flight evolution debate. We demonstrate that the ventricle in tinamous is the smallest among all other bird families, 0.30% on average, much smaller than the average ventricular size for all other bird families, 1.1%. The smaller ventricular size is correlated with differential gene expression in MAPK terminal kinases in the Ornate Tinamou (No) and the Chilean Tinamou (Np) in comparison to the Red Junglefowl (Gg). In the same line, the growth pattern of the ventricles in tinamous is comparable to the American alligator, an archosaurian reptile. The small ventricular size significantly reduced cardiac output in No without limiting perfusion pressures, which were similar to Gg values in anesthetized individuals (121±18 mmHg in No vs. 98±7 mmHg in Gg). When challenged to fly and run, lactate values increased significantly more in No (15.7 mM) and Np (18.7 mM) in comparison to Gg (9.2 mM) and excess post-exercise oxygen consumption was 6-fold larger in both tinamou species. Body temperature regulation was also impaired in No and dropped significantly by more than 1°C for longer than 2 h post-challenge. All these features support the idea that the small heart of tinamous is a plesiomorphic trait and that their ground-up, short and not sustained flapping flight represents an intermediate step in the evolution of the aerobic sustained flapping flight of modern birds. This evidence lead us to formulate a new hypothesis, the “heart to fly”

48 hypothesis that states that sustained flapping flight in modern birds required an enlargement
49 of the heart for the aerobic performance of the flight muscles.

50

INTRODUCTION

The evolutionary origin of avian flight has been long a matter of scientific debate without unanimous agreement ([1-3] to mention a few of the latest studies). Flight could have appeared already in paravians as *Anchiornis* [4], in the most ancient true birds such as *Archaeopteryx* [5] or in more advanced taxa such as Ornithuromorpha [6]. Flight requires a suite of morphofunctional traits to steer and coordinate such locomotory pattern, ***i.e.*** feathers, muscles and the skeleton. These features have been studied in many of the Mesozoic fossils in the last two decades to paint a complex scenario in which late dinosaurs and early birds experimented with different airborne gliding or flying behaviors [7]. Ultimately, sustained flapping flight became the successful alternative adopted by birds to be subsequently refined by gliding or hovering in some species. These conclusions have been reached from fossilized specimens and biomechanical models derived from them [8-11]. Therefore, these conclusions rely on the implicit and unspoken assumption that other physiological processes required for flight must have evolved in tune with flight biomechanics.

Flight is an energetically costly activity and the cardiovascular system is of crucial importance to support the elevation in metabolism associated with it [12]. Unfortunately this basic tenet has not been considered in the discussion of flight evolution, most likely because soft tissues fossilize poorly and data on cardiovascular variables cannot be directly measured. Relevant progress, however, has been made in other fields and neontological studies on extant bird species focusing on postnatal locomotor development [13, 14] or limb functional morphology [15] are providing substantial arguments to interpret the evolution of avian flight.

74 Heart size and maximal metabolic rate scale similarly to body mass, which suggests
75 that cardiac size limits maximal aerobic scope and, consequently, maximal metabolism
76 during flight [16]. Therefore, our main argument is that evolutionary modifications in the
77 cardiovascular system would have been critical to support the high aerobic demand of
78 sustained flight and that a suitable musculoskeletal arrangement is not sufficient to prove
79 flight capabilities. Thus, the appearance of flapping flight cannot be unequivocally coupled
80 to sustaining aerobic flight unless the cardiovascular system made it possible.
81 For this purpose we chose several species from the tinamou family because there is
82 circumstantial evidence that they have the smallest heart among birds [17-19], about 0.3%
83 of body mass, which is several times smaller than in phasianids (1.03%), an ecologically
84 convergent group (summarized graphically in Sup.Fig.1). Tinamous are the only living
85 flying paleognaths, a clade shared with the flightless Ratites (ostriches, emus, rheas,
86 cassowaries and kiwis). In the evolution of Neornithes or "modern birds", Palaeognathae
87 split from Neognathae (all the other living birds) in the Cretaceous [20-22]to name a few
88 supporting studies). Tinamous only perform short and burst flapping flights initiated from
89 the ground [23, 24] and it has been argued that the small heart limits flight [25].
90 We devised our study to evaluate the physiological performance of the heart in
91 tinamous; to confirm and unequivocally document the ontogeny of cardiac growth and its
92 adult morphometry; and to provide evidence of putative gene regulatory pathways involved
93 in the architecture of cardiac size. Therefore, we used two tinamou species from the same
94 genus, the Ornate Tinamou (*Nothoprocta ornata*) found in the Andean Highlands of Peru,
95 Bolivia, Argentina and Chile (altitude range 2500 - 4800 m) and the Chilean Tinamou
96 (*Nothoprocta perdicaria*) found in lowland areas in Chile. These species were compared
97 with Red Junglefowl (*Gallus gallus*) a neognath species with a larger heart size but a

98 similar flight mode, i.e. a short not-sustained flapping flight. We also included data from an
99 outgroup species, the American alligator (*Alligator mississippiensis*) to analyze the
100 comparative ontogeny of cardiac growth. Crocodilians are the only extant non-avian
101 archosaurs and have a typical reptilian small heart [26, 27].

102 Our hypothesis was that the relative small size of the heart in tinamous would
103 restrict their aerobic performance and thermoregulatory capacity, and if the small size of
104 the heart is phylogenetically related to reptiles, this could be observed in their ontogenic
105 development. This multi-level and integrated approach intends to explore proximal and
106 ultimate causes for a morphological trait in living birds that has important implications for
107 the interpretation of the evolution of avian flight.

108

109 **RESULTS**

110 We characterized cardiac growth using power allometric equations ($VM = a\ BM^b$
111 where VM is ventricular mass and BM is body mass) to the data for each species (Figure 1).
112 The equations differed significantly in the mass exponent between Red Junglefowl and the
113 tinamous and the alligator. For an individual with a body mass of 700g, relative ventricular
114 mass would be 0.21% in the alligator and the Ornate Tinamou, 0.24% in the Chilean
115 Tinamou and significantly larger (0.42%) in the Red Junglefowl. The emerging pattern
116 from the data is that cardiac growth in tinamous and alligators follows a similar trajectory
117 while in Red Junglefowl cardiac growth is increased throughout life. The differences are
118 not apparent during early development and the cardiac growth curves start splitting at a
119 body mass approximately above 80 g, which corresponds to an approximate age of 2-4
120 weeks.

Ornate Tinamous had the smallest relative ventricular size of all three species with a ventricular index of 0.24% (SD 0.03, N=45) followed by Chilean Tinamous (0.28% SD 0.04, N=40) and Red Junglefowl, with the largest ventricular sizes and a significant difference between males and females (0.42% SD 0.05, N=70 and 0.36% SD 0.05, N=68 respectively) as shown in Figure 2A. The small ventricular size can be generalized to the entire family Tinamidae regardless of habitat or altitudinal distribution (Table 1). Data on ventricular mass from 13 species from both tinamou subfamilies gives a consistently small ventricular mass, ranging between 0.34% in the Small-billed Tinamou *Crypturellus parvirostris* and 0.15% in the Great Tinamou *Tinamus major*. This pattern is clearly evident when accounting for relative heart size in all the bird species measured to date as compiled by Nespolo and the authors [28] and graphically shown in Suppl.Figure 1.

Relative right ventricular mass was also highest in Red Junglefowl (21.8% SD 2.3, N=38) than in both tinamous (16.9 % in Ornate Tinamou and 15.7% in Chilean Tinamou) as shown in Figure 2B. Despite having smaller hearts, the left ventricular wall was thicker in tinamous than in Red Junglefowl (Figure 2C). In Red Junglefowl, the left ventricular wall accounted for 23% (SD 4, N=10) of the diameter of the heart and this was larger in the Chilean Tinamou (25%, SD 2, N=14) and even larger in the Ornate Tinamou (33%, SD 5, N=22). These values are comparable to those obtained in ethanol preserved hearts from other tinamou species, which ranged from 25% in the White-bellied Nothura *Nothura boraquira* to 32% in the Small-billed Tinamou *Crypturellus parvirostris* (Figure 2D).

Measurements of wall thickness using echocardiography in conscious tinamous are comparable to the morphometric measurements. They are not equivalent because they were not performed at the same anatomical landmark. Left ventricular wall thickness was not significantly different between Ornate Tinamous and bantam chickens (21% in the tinamou

vs. 17% in the bantam chicken (p=0.08) in diastole; 37% vs. 32% respectively (p=0.12) in systole, Figure 3A). Fractional shortening in conscious animals (Figure 3B) and heart rate in conscious (Figure 3C) or anesthetized animals (Figure 4B) did not differ either and mean arterial pressure was comparable between Red Junglefowl and Ornate Tinamou (Figure 4A). Mean arterial pressure was 115 mmHg (SD 30) in Ornate Tinamou and 136 mmHg (SD 30) in Red Junglefowl. Heart rate was 259 beats per minute (SD 65) in Ornate Tinamou and 295 beats per minute (SD 52) in Red Junglefowl. Isoprenaline, a beta-adrenergic receptor agonist, at a dose of 3 ug kg^{-1} did not significantly stimulate cardiac function but a highly significant difference in cardiac output and stroke volume was observed (Figure 4CD). Cardiac Output was only 98 ml min^{-1} kg^{-1} (SD 37) in Ornate Tinamou. It was 2.8 fold larger in Red Junglefowl (276 ml min^{-1} kg^{-1}, SD 84). Stroke volume was 0.37 ml kg^{-1} (SD 0.08) in Ornate Tinamou and 2.5 fold larger in Red Junglefowl (0.93 ml kg^{-1}, SD 0.17). Altogether, the results point out that the small size of the tinamou heart limits cardiac output but not the capability of the heart to generate pressure.

Resting metabolic rate 70-90 min after an aerobic challenge did not differ between species: 1.05 mlO_2 g^{-1} h^{-1} (SD 0.07 N=5) in Red Junglefowl (Figure 5A) vs. 0.91 mlO_2 g^{-1} h^{-1} (SD 0.07 N=10) in the Chilean Tinamou (Figure 5B) and 0.98 mlO_2 g^{-1} h^{-1} (SD 0.18 N=6) in the Ornate Tinamou (Figure 5C) but excess post-exercise oxygen consumption (EPOC) was almost 6-fold larger in both tinamou species. EPOC averaged 297 mlO_2 kg^{-1} in tinamous and was only 50 mlO_2 kg^{-1} in Red Junglefowl (Figure 5D). This is also reflected in the lactate values reached after the aerobic challenge (Figure 5E), which were significantly higher in tinamous (18.7 mM SD 1.7, N=8 in Chilean Tinamou and 15.7 mM SD 0.9, N=16 in Ornate Tinamou) than in Red Junglefowl (9.2 mM SD 1.5, N=6).

Cloacal temperatures were more labile in the Ornate Tinamou than in the Red Junglefowl and increased significantly after the chase-challenge (Figure 6). In the colder environment (5 °C) cloacal temperature was lower than in the warmer thermoneutral environment (25°C) only in the tinamou, 39.9°C (SD 0.2 N=6) vs. 40.4°C (SD 0.3 N=6). Forty minutes after the chase-challenge cloacal temperature had dropped significantly by 1°C at both thermal environments, to 38.5°C (SD 0.5 N=6) at 5°C and to 39.5°C (SD 0.6 N=6) at 25°C, and body temperature remained low even 2h after the challenge. The lability in body temperature was not seen in Red Junglefowl (Figure 6 and Suppl. Figure 2).

To further explore the heart size differences we focused on the gene expression of four kinases in the PI3K/Akt and MAPK signaling pathways: ERK (gene *ERK2*), JNK (gene *JNK1*), p38 (gene *p38*) and PI3K (gene *PIK3CA*). We found a consistent downregulation in *ERK2* (Figure 7A) and an upregulation in *p38* (Figure 7B) in both tinamou species in relation to the Red Junglefowl. *ERK2* was downregulated down to 14% of the Red Junglefowl values in both species and *p38* was upregulated 10-fold and 14-fold in the Chilean Tinamou and the Ornate Tinamou respectively. *JNK1*, on the other hand, was significantly 5.5-fold upregulated in the Chilean Tinamou, but only 1.8-fold upregulated in the Ornate Tinamou not reaching statistical significance (Figure 7C). No significant differences were found for *PIK3CA* (Figure 7D).

DISCUSSION

The tinamou heart can generate pressure but not flow

Our results confirm that the heart of tinamous is the smallest among all extant bird species [25, 28]. This is true for all thirteen tinamou species studied to date (by us or by

192 others) and the inter-specific variation is not related to altitudinal or latitudinal geographic
193 distribution, habitat or their belonging to the subfamily Tinaminae or Rhynchotinae (Table
194 1). The small heart size inevitably limits its functional output and mass specific cardiac
195 output and stroke volume in the Ornate Tinamou are less than half the chicken values
196 (Fig.4C-D), the values obtained in neognathous species [29], and even in other
197 palaeognathous birds such as the emu [30]. Such low stroke volumes (0.34-0.4 ml min^{-1} kg^{-1})
198 are comparable to those in alligators [31] but the faster heart rates in tinamous account
199 for five-fold larger cardiac outputs.
200 Cardiac output is limited but cardiac contractility is not compromised. Fractional
201 shortening is high and comparable to equivalent measurements in bantam chickens (Fig.3B)
202 and White Leghorn chickens [32]. The left ventricle of the Ornate Tinamou is competent to
203 generate bird-like mean arterial pressures (above 120 mmHg, Fig.3A) while maintaining
204 bird-like heart rates (above 290 min^{-1} during anesthesia and above 380 min^{-1} in conscious
205 animals, Figs.4B and 3C respectively).
206 The novel observation of crocodilian-like stroke volumes and reduced cardiac outputs
207 combined with normal arterial pressures is a key finding for a bird species and provides the
208 first clue of the primitivism of tinamous. We sustain that the tinamous achieved a high
209 systemic pressure taking advantage of the thickening of the left ventricle (Fig.2C) without
210 an enlargement of the cardiac chambers. Because of the small chamber size, a modest
211 thickening of the left ventricular wall would facilitate the development of higher pressures
212 without incurring in excessive wall tensions as predicted by the principle of Laplace [33].
213 Bantam chickens at altitude also displayed a thicker left ventricular wall (Fig.3A) but this is
214 due to a hypertrophic response to altitude acclimatization [34].
215

The cardiac morphology and physiology of the tinamous limits aerobic performance

Ventricular size and its flow-limited capability have no effect on resting metabolism in thermoneutral conditions. Oxygen consumption of the Ornate Tinamou and the Chilean Tinamou is not different from the measures in Red Junglefowl under similar experimental conditions (close to 1 ml O_2 g^{-1} h^{-1}). Our values for Chilean Tinamous are 32% higher than those obtained previously [35], but they are more robust considering sample sizes and the fact that there was an agreement between the two tinamou species. The values contrast with the lower resting metabolism of Ratites, which is likely due to the reduction in pectoral muscle and the loss of flight instead of phylogeny, as pointed out in large interspecific studies of metabolism [36, 37]. Our metabolic measurements are consistent with the fact that tinamous have well developed pectoral muscles [38], with percentage values of pectoral mass ranging between those in many other bird families as shown in Supplementary Figure 3.

Albeit not in resting conditions, the small heart in tinamous is a relevant hindrance when demand for oxygen supply increases. When both tinamou species are challenged to fly and perform above resting levels, they reach exhaustion quickly while significantly accumulating lactate because during this period the heart is unable to supply enough oxygen to the body. Large increases in lactate are also observed in restrained estuarine crocodiles with slow recoveries [39]. Aerobic metabolism after the exhaustion challenge, the so called post-exercise oxygen consumption (EPOC) [40] was also remarkably long-lasted (60 min) for tinamous but almost absent for Red Junglefowl, despite the fact that lactate also increased in the latter. Lactate oxidation is not the main metabolic burden post-exercise [41] so the main conclusion from our results is that the larger lactate release and the significant EPOC response reflect that the exhaustion challenge requires an anaerobic

240 contribution that is also considerably longer than what is seen in mammals (17 min in sprint
241 wheel running in mice for example [42]). From a comparative point of view, the tinamou
242 EPOC could be considered more reptilian than avian [43] and we infer that the cardiac
243 performance of tinamous upon aerobic challenges is reptilian-like, once again supporting
244 the primitivism of tinamous.

245 The aerobic challenge also imposed limitations to the ability to regulate body
246 temperature. Basal cloacal temperatures in the Ornate Tinamou are clearly bird-like albeit
247 on the lower part of the range [44]. Body temperature drops below resting values for a
248 period longer than 2h and the effect is enhanced at lower ambient temperatures. The
249 striking heterothermic swing (rise in body temperature during exhaustion and drop
250 thereafter) is likely magnified by the experimental conditions where the animals could not
251 use behavioral thermoregulation but it was not seen in bantam chickens, which attests to the
252 physiological difference between species. To distinguish if this observation is an adaptive
253 energy-sparing mechanism or a maladaptive consequence of hyperventilation-driven body
254 cooling further studies are needed, but the body temperature of the Ornate Tinamou clearly
255 shows greater variations than chickens and also a larger dependence on ambient
256 temperatures.

257 It is important to emphasize that the physiological limitations ascribed to a smaller
258 heart mainly apply to challenging scenarios such as the exhaustive bouts of exercise
259 imposed experimentally or the explosive burst of flight experienced in the wild when
260 escaping from potential predators, which could be the most significant aerobic challenges a
261 tinamou is exposed to attending to the EPOC measured [43]. In resting conditions tinamous
262 are not metabolically challenged and remain thermally stable. The flight performance in
263 tinamous is highly conditioned by their small heart and this could explain why tinamous

avoid flying as much as possible. Tinamous prefer to escape from predators walking and running and use their cryptic plumage and secretive habits as the main antipredatory mechanism. When they do not have any other option but flying, they do it in a burst-like manner with a powerful jump. Such flapping flight, normally accompanied with a strident vocalization, can be performed two or three consecutive times before the bird is unable to fly again and it is possible to hand-catch them [23, 45] and our own field observations). We call this type of flight as non-sustained flapping flight (NSFF) with the objective to differentiate from the aerobic sustained flapping flight (SFF) that is observed in the majority of neognathous birds.

The ontogenic development of the tinamous' heart show a clear signal of primitivism

The ontogenic development of the heart of both tinamou species differs widely from that of the Red Junglefowl but otherwise is remarkably alike that of the alligator. Estimated relative ventricular mass at adulthood is 0.21-0.24%, similar to alligator published values [26, 27]. Thus, we speculate that the heart in neognathous birds display a larger proliferative activity that would yield a bigger heart. The similarity between cardiac growth between tinamous and alligators but not Red Junglefowl is hard to ascribe to a casual or convergent process, and the most parsimonious interpretation is that the neognathous larger heart is an evolutionary novelty in comparison to the tinamou heart that is more reptilian like and could be assumed as primitive.

The genetic mechanisms behind the differential cardiac growth in bird species are mostly unknown so we singled out PI3K/Akt and MAPK pathways as a first approach

288 because of their prominent role in cardiac development and plasticity in mammals [46, 47].
289 MAPK activation, mainly ERK, also regulates cardiogenesis and differential cell lineage
290 growth in the embryonic chicken heart [48]. The expression pattern that we report for the
291 three terminal MAPKs and for PI3K is consistent with the knowledge in mammalian
292 myocardial tissue [47]. We speculate that the observed ERK upregulation in Red
293 Junglefowl promotes cardiac growth resulting from growth-factor mediated physiological
294 hypertrophy [47]. On the other hand, the stress-activated kinases, p38 and JNK, are either
295 upregulated or do not change in tinamous, which is congruent with the antagonistic effects
296 reported for both JNK [49] and p38 [50] on mammalian cardiac growth. Altogether, a
297 reduced ERK expression and an enlarged JNK/p38 expression of tinamous may be a
298 signature of a poor cardiac proliferative capability in primitive birds, speculations that
299 require further studies.
300
301 **The "heart to fly" hypothesis on the acquisition of sustained flapping flight in**
302 **Neornithes: "sustained flapping flight requires a large heart for the aerobic**
303 **performance of the flight muscles"**
304
305 Based on all our evidence, we propose that the small heart size in tinamous is a
306 plesiomorphic trait for all Neornithes which was likely shared with archosaurian ancestors.
307 The lack of fossil evidence for dinosaurian or primitive avian hearts prevents direct
308 inferences as to the size of their hearts [51, 52] but the evidence that several avian
309 characteristics were acquired progressively along avian evolution, for instance a
310 unidirectional air ventilation and high metabolic rates [6], supports our interpretation that
311 novel metabolic demands imposed a strong adaptive pressure on the reptilian heart to

312 increase blood systemic pressure and metabolic rates and to perform some kind of flapping
313 flight, more like a NSFF as the first step.

314 Support for the plesiomorphy of the small heart comes from the fact that among all
315 neognathous birds, no reduction of the flight capacity has been associated with a reduction
316 in heart size [28] despite the fact that pectoral muscle mass is actually reduced [36, 37]. The
317 alternative evolutionary scenario in which the small tinamou heart derived from a larger
318 heart in ancestral species with good flight capabilities cannot be fully discarded but is less
319 parsimonious. If a small heart and NSFF are derived characters, what selection pressures
320 could be behind? Note that tinamous need to constantly use cryptic behaviors that restrict
321 their foraging and reproduction niches, they are unable to use flight for migration or
322 dispersion, and they are highly susceptible to predation when exhausted. None of these
323 seems to favor a positive selection pressure for a secondary acquisition of a small heart.

324 Along the phylogenetic history of birds, the acquisition of a well-developed pectoral
325 musculature (inferred from osteological characters, *i.e.* the progressive increase of the
326 carina in the sternum) has been directly coupled to the capacity for powered flapping and
327 sustained flight, and some authors have even ventured that birds without carina and pectoral
328 musculature such as *Archaeopteryx* could have displayed powered flapping flight [5, 11,
329 53]. Our physiological data does not support the claim that avian species preceding
330 Neornithes would have been capable of sustained flapping flight (SFF). Ruben (1991)
331 actually proposed that an anaerobic reptile-like mode of muscular performance would be
332 enough to let *Archaeopteryx* to take flight from the ground and to have a prolonged SFF
333 [11]. We provide evidence that a high aerobic capacity is required for SFF based on the fact
334 that tinamous are metabolically limited and cannot perform SFF.

335 Tinamous are ground-dwelling birds that are able to take off from the ground and
336 display NSFF [23, 45]. All the osteological and muscular machinery of tinamous belong to
337 a Neornithine flying bird, but our results show that even in the presence of a big carinated
338 sternum that supports enough pectoral musculature (Supplementary Figure 3), a small heart
339 limits aerobic scope and is not compatible with SFF. Although in general terms NSFF is
340 present even in some Neognathous birds as for example phasianids, the metabolic
341 characteristics differ widely as our results in the Red Junglefowl show.
342 The evolutionary way in which the "modern bird flight" appeared in the avian lineage
343 from their reptilian ancestors has been heavily debated for decades [1-3, 54]. The trees-
344 down flight hypothesis considers that the powered flight capacity was acquired from
345 arboreal gliding animals. In accordance with this point of view flapping flight would have
346 preceded the ability to initiate flight from the ground. An alternative hypothesis proposes
347 that powered flight first required the capacity to take up the flight from the ground. Our
348 hypothesis fits better with the second point of view and is not in conflict with the fact that
349 several fossil non-Neornithes avian lineages would have been arboreal or gliders because
350 animals without an adequate aerobic physiological capacity could have taken the advantage
351 of climbing trees or reaching other elevated points in order to glide but not to perform a
352 SFF. In this situation the control of gliding and landing could explain the early evolution of
353 complex wings and feathers, including the presence of the alula [55-57].
354 With the evidence that the cardiac morphology and physiology of tinamous is not
355 suited for sustained flight we propose a new hypothesis on the acquisition of flight in
356 Neornithes. The hypothesis, which we call the "heart to fly" hypothesis states that sustained
357 flapping flight requires a large heart for the aerobic performance of the to the flight muscles.
358 In consequence, the NSFF of tinamous is primitive and represents the intermediate flight

359 strategy between the non-Neornithes avian fossils and the modern neognathes. In this
360 scenario, the typical jumping take-off from the ground followed by the NSFF that tinamous
361 perform today is a reflex of the putative first type of flight performed by Neornithes, point
362 of view that is in agreement with the neontological-based proposals of the evolution of the
363 avian flight [14, 58].

364 Based on the new hypothesis we envision the evolutionary history of modern birds as
365 two different paths from an ancestor with a small heart and NSFF, much like the extant
366 tinamous. The first path is the one exemplified by Ratites, in which the loss of the flight
367 capacity (including the loss of all the pectoral musculature and their skeletal support) was
368 aimed at optimizing cursorial abilities for better foraging and predator evasion. The second
369 path is the one followed by Neognaths in which SFF was possible because of the larger
370 heart and the suitable musculoskeletal organization were already in place. Only at this point,
371 the adequate increase in stroke volume and cardiac output allowed for the real conquest of
372 the air for modern Neognaths, and from here the evolution of other types of flight as gliding,
373 soaring, hovering and even returning to short flapping flights and flightlessness.

374 Two potential conflicts with the "heart to fly" hypothesis require further discussion,
375 namely the larger heart mass of Ratites and the assumed flight capabilities of Lithornitids,
376 an extinct paleognath clade.

377 First, the heart in the few Ratites species studied to date, i.e. Ostrich, Emu and
378 Greater Rhea is not small and fall in the range of most Neognath families (Suppl. Figure 1).
379 Because Ratites do not fly we propose that cardiac enlargement in birds occurred
380 independently more than once. For ratites the selective pressure could have been the need
381 for running endurance. Ostriches, for example, are acknowledged as the fastest bipeds with
382 the largest capacity for long-endurance running [59]. Emus, on the other hand, have aerobic

383 scopes in the range of 11-36 times basal metabolic rates while running [30, 60], values that
384 are higher than the aerobic scopes measured in flying birds [16, 60]. Based on their
385 nocturnal lifestyle and sedentarity, it is tempting to speculate that kiwis may have a small
386 heart but data is missing.

387 Although all modern phylogenies support the early divergence of paleognaths in the
388 evolution of Neornithes, there are differences in the proposed phylogenetic relationships
389 between ratites and tinamous. Morphological phylogenies support an early divergence of
390 tinamous followed by ratites as a derivate monophyletic group [20, 61-64]. This scenario is
391 in line with our hypothesis of the plesiomorphy of tinamou traits and implies the secondary
392 loss of flight in ratites. Molecular phylogenies, on the other hand, nest tinamous inside
393 ratites [21, 65-71]. At first sight, this could imply that tinamous acquired flight secondarily
394 from flightless ancestors but we find it highly unlikely. Our suggestion, which is
395 compatible with molecular phylogenies, is that the ancestral paleognath was capable of
396 flight (a NSFF type) and lost it multiple times in different Ratite lineages [72]. Living
397 tinamous, descendants of some line of flying paleognaths, conserved NSFF and their
398 ancestral cardiovascular traits.

399 The second potential conflict relates to the flight capabilities of extinct paleognaths
400 inferred the dispersed geographical location of fossil findings. The more recent molecular
401 calibrations date the origin of Neornithes and the splice of Palaeognathae and Neognathae
402 in the Late Cretaceous [21, 73], estimations congruent with the scarce fossil record of this
403 period [74]. Although neognath fossils are best preserved [75, 76], fragmentary postcranial
404 material of *Iaceornis* [77] could represent the earliest palaeognath present in the Late
405 Cretaceous [74], but no solid fossil evidence of a Cretacic palaeognath with NSFF exists.
406 The first paleognath fossils are from the Paleocene (60-66 million years ago) and

correspond to flightless ratites from Europe and South America [78, 79]. Tinamous appear in the fossil record much later, in the early Miocene, 16.5 million years ago [80]. The best preserved non-Ratite palaeognathae fossils are the medium-sized volant Lithornithiforms known from the Paleocene and Eocene layers of North America and Europe [81, 82]. They are taxonomically rooted with all modern birds, either as a sister taxa of all the Neornithes [20, 82], as sister taxa of all other paleognaths [72] or as a sister taxa of tinamous [64, 80, 83]. Based on morphological traits from fossil findings, Lithornithiformes were described as capable of sustained flight [81], and this suggestion was later used to justify the outcomes of recent molecular paleognath phylogenies that place for example New Zealand kiwis as the closest relatives of Madagascar elephant birds, or South American tinamous clustering with New Zealand moas [69, 70, 72, 84]. Counter to this argument, the distribution of several tetrapod fossils is congruent with the presence of ephemeral land bridges in the Late Cretaceous between continental land masses [85, 86] and geologic evidence show that it was possible [87]. Then, the possibility that hypothetical ancient palaeognath birds with tinamou-like cardiovascular physiology and NSFF dispersed walking widely in Late Cretaceous and Early Cenozoic [88] could explain the known distribution of fossil and current palaeognathae in agreement with our hypothesis.

Our results highlight the crucial importance of physiology, specifically cardiac physiology to understand more completely the evolution of the avian flight. The mechanical and aerodynamic interpretations inferred from the fossils need to consider that this machinery needs a power source, the heart, and that final flight performance depends on it.

MATERIAL AND METHODS

Animals Adult Ornate Tinamous *Nothoprocta ornata* for the physiological experiments were born in captivity from a founding group of captured wild birds or artificially incubated wild eggs obtained in the surroundings of the town of Qurpa, Bolivia (3800 meters above sea level). Animals were hold at the animal facilities in the Cota-Cota campus (3420 m, Universidad Mayor de San Andrés, UMSA, La Paz, Bolivia) in 8m^2 pens holding up to 5 animals per pen and exposed to natural conditions. Animals were fed *ad libitum*. Adult and juvenile Ornate Tinamous for the anatomical studies were hunted with shotgun mainly during the dry season (May to July) in several localities in the Bolivian high plateau between 3800 and 4300 m. Hunting and animal maintenance were carried under the permission for scientific studies from the Bolivian General Direction of Biodiversity and Protected Areas (DGBAP). Chilean Tinamous *Nothoprocta perdicaria* were obtained from Tinamou Chile SL, a farm located in the city of Los Angeles (140 m, VIII Región del Bío-Bío, Chile). Adult individuals at the farm were kept in mixed reproductive groups in 12 m^2 pens. Young individuals were kept in reduced groups (up to 10 individuals) in 0.5 m^2 square holding boxes under tungsten filament bulbs used for heating. Adult Red Junglefowl *Gallus gallus* were kept at the research chicken house of the University of Linköping (Ljungsbro, Sweden, 71 m) in 22m^2 indoor pens at 19°C and under 12:12h light:dark cycle. Food was provided *ad libitum*. The population has been kept under non-selected captive conditions since 1993 [89]. Young individuals were kept at the hatchery on the university campus in individual pens at 28°C and under 12:12 light:dark cycle with food provided *ad libitum*.

Adult domesticated chickens of a bantam breed were purchased from a common market in the city of La Paz, Bolivia, and used in experiments where comparisons under

similar environmental conditions were deemed relevant. Bantam chickens are less prone to altitude-related syndromes (pulmonary hypertension and ascites) due to their smaller size and slower growth and were preferred over meat or proper egg-laying breeds. Animals were kept in the same facilities than the Ornate Tinamous at UMSA, La Paz, Bolivia.

We also used preserved specimens from 8 tinamou species: the Great Tinamou *Tinamus major* (N=2), the Undulated Tinamou *Crypturellus undulatus* (N=2), the Tataupa Tinamou *Crypturellus tataupa* (N=1), the Red-winged Tinamou *Rhynchotus rufescens* (N=3), the Ornate Tinamou *Nothoprocta ornata* (N=2), the Andean Tinamou *Nothoprocta pentlandii* (N=2), the White-bellied Nothura *Nothura boraquira* (N=2), and Darwin's Nothura *Nothura darwinii* (N=3). Specimens were collected for skin preservation purposes under an experimental hunting permit from the Bolivian General Direction of Biodiversity and Protected Areas (DGBAP). The bodies were preserved in ethanol 90% and deposited in the Colección Boliviana de Fauna, La Paz, Bolivia.

Ethical considerations

Experiments in Bolivia were carried out under ethical license from the Animal Research Ethical Committee of the Bioethical National Board (CEI-CNB) issued in December 2011. Experiments in Sweden were carried out under ethical permits granted to J. Altimiras by the regional ethical committee of Linköping (Dnr.25-10, 26-10, 19-11 and 9-13).

Heart Morphometry

Animals were killed by gunshot or euthanized by decapitation. Body mass was immediately obtained with a spring scale and the heart was dissected out, rinsed in 0.9% NaCl, immediately placed in cold cardioplegic solution (in mM: 40 NaCl, 100 KCl, 2

479 Ca_2Cl, 1.8 K_2HPO_4, 10.1 Na_2HPO_4, pH=7.4) to arrest the heart in diastole and kept in a
480 cold environment. Within 6 h post-mortem the hearts were dissected to remove the central
481 outflow tract, the atria and the fat deposits at the atrio-ventricular boundary and the
482 ventricles were blotted dry before weighing on a digital scale down to 0.01g resolution.
483 Right ventricular mass was obtained in a subset of hearts by weighing the free right
484 ventricular wall after dissection. The conspicuous muscular right atrioventricular valve
485 characteristic of bird species [90, 91] was also included as part of the right ventricular mass.
486 The other subset of hearts was used to measure wall thickness by embedding the
487 ventricles in cryo-medium (Tissue-Tek O.C.T., Sakura Finetek Europe, Leiden, the
488 Netherlands) and freezing and cutting in a cryostat (Microm HM 550, Thermo Fisher
489 Scientific, Walldorf, Germany) in 500 micrometer sections as previously reported [92].
490 Prior to freezing both ventricles were filled with a volume of cryo-medium corresponding
491 to half of the calculated stroke volume calculated from allometric equations ($SV = 0.175$
492 $HeartMass^{1.05}$, [93]. In preliminary tests we observed that a volume of cryo-medium
493 equivalent to the calculated stroke volume caused the right ventricular wall to rupture at the
494 time of freezing so the volume was halved to avoid wall rupture while keeping a clear
495 definition of the internal chamber diameter.
496 All cryostat sections were photographed directly on the specimen holder of the
497 cryostat at 20x magnification by mounting a USB Mediscope camera (Optilia Instruments
498 AB, Sollentuna, Sweden) in the cryostat freezing chamber. Images from each heart were
499 calibrated by placing a metallic circle of known dimensions on the specimen holder at the
500 start and the end of each session. Particular care was placed in aligning the heart on its long
501 axis before freezing to insure non-skewed cross sections. Right and left ventricular
502 thickness was taken from the last two consecutive and most caudal sections in which the

503 right atrioventricular valve (RAV) was still visible as graphically depicted in
504 Supplementary Figure 4. Unlike in mammals, the RAV in birds is muscular [90] and it is
505 very apparent in cross-sections. The thickness of the right and the left free ventricular walls
506 in each section was determined by averaging 10 single measurements that spanned the
507 entire free wall in each section using NIS- Elements Advanced Research software (Nikon
508 Instruments). To normalize for differences in heart size all measurements of wall thickness
509 were made relative to the diameter of the heart in the respective sections. The diameter was
510 estimated geometrically from the total area of the section assuming a circular shape. Hearts
511 from ethanol preserved tinamou specimens of different species were sectioned directly by
512 hand and measurements of ventricular wall thickness were done from images in the same
513 manner as above.
514 Ventricular mass data at different ages and stages of development (from embryonic
515 age to an adult mass of 700 g) was collected from different species. Data on tinamous was
516 collected for the purpose of this work. Data from Red Junglefowl and alligator was
517 compiled from other studies from our group [94-99].
518
519 **Echocardiography in conscious birds**
520 We used portable ultrasound equipment (LogicScan 64 FLT-1T, Telemed, Vilnius,
521 Lithuania) to image the heart in conscious birds in a right parasternal short-axis view as
522 previously described [100] using a 9 MHz linear probe (HL9.0/40/64D, Telemed, Vilnius,
523 Lithuania). Wall thickness of the free left ventricular wall was measured using Echo Wave
524 II software (Telemed, Vilnius, Lithuania) by focusing the probe on the transverse section
525 where the right ventricular chamber was no longer apparent as previously described [100].
526 We used tonic immobility as a way to avoid anesthesia and obtain data in conscious

individuals. Tonic immobility was induced by restraining a bird on its back for 15 s and releasing the pressure exerted by the hand gently [101]. Ornate Tinamous went readily into tonic immobility after one or two inductions while chickens required a maximum of five induction attempts. Although tonic immobility can be maintained for long periods and it is not harmful to the animal [102], it was kept only as long as needed for the procedure, typically under 5 min.

Cardiovascular function in anesthetized animals

Anesthesia was induced in a plastic box with 4% isoflurane provided by a vaporizer (Tec 3, Ohmeda). Once the animal lost equilibrium it was placed on a heating pad with a loose plastic mask suppling the anesthetic gas mixture (1% Isoflurane:Oxygen) at a rate of 40 ml min^{-1}. Ventilation rate and heart rate were continuously monitored from subcutaneous electrodes using an impedance converter (Model 2991, UFI, Morro Bay, California, USA) connected to a Powerlab amplifier (Model 4/35 ADInstruments-Europe, Oxford, UK) and the data was logged in a computer using LabChart 7Pro software (ADInstruments-Europe, Oxford, UK). Body temperature was monitored using a cloacal probe connected to the same monitoring system via a temperature pod (T-type Pod ML312, ADInstruments-Europe, Oxford, UK) and maintained at an average of 40°C for Red Junglefowl and 39°C for Ornate Tinamous with the use of the heating pad.

After a surgical plane of anesthesia was achieved by adjusting isoflurane concentration in the breathing mask we proceeded to catheterize the ulnar artery in the right wing with a polyethylene catheter (PE-90, 1.27 mm external diameter, 0.86 mm internal diameter, Clay-Adams Intramedic, New York, USA) pulled to a thinner tip. The tip was advanced a length of 10 mm into the artery in an upstream direction and secured in place

551 with sutures. The catheter was then coupled to a disposable blood pressure transducer
552 (DPT610, Peter von Berg Medizintechnik GmbH, Eglharting, Germany) connected to a
553 bridge amplifier (FE221 ADInstruments-Europe, Oxford, UK) and to the same Powerlab
554 amplifier and recording system. Access to the aorta to record cardiac output required the
555 opening of the interclavicular air sac but this interfered with gas anesthesia because a stable
556 control of inhaled isoflurane concentration could no longer be achieved. While the animal
557 was still anesthetized with isoflurane we switched to injectable anesthesia with a mixture of
558 ketamine (20 mg kg^{-1}) and xylazine (5 mg kg^{-1}) administered intraperitoneally. Once the
559 first injection took effect we removed isoflurane from the breathing gas and we injected a
560 second dose of anesthesia to achieve a final dose of 40:10 ketamine:xylazine. After the new
561 anesthesia took full effect we opened the interclavicular sac, identified the aorta after the
562 branching from the right brachiocephalic artery, freed it from connective tissue and placed
563 a perivascular transit-time Doppler flow probe (H3MB 3mm, Transonic Systems Inc.,
564 Ithaca, New York, USA) around it. The probe was connected to a flowmeter (T106,
565 Transonic Systems Inc., Ithaca, New York, USA) and this to the recording system.
566 The whole operation up to this point would typically take 60-90 min after which the
567 animal was monitored for stable cardiovascular parameters for at least 15 min. We later
568 proceeded with a bolus injection of saline solution (1 ml kg^{-1}) to discard volume effects
569 from the subsequent injection of isoproterenol, a beta-adrenergic agonist, at a dose of 3 ug
570 kg^{-1}. Fifteen minutes after injection of isoproterenol the animal was euthanized by
571 decapitation.
572 Blood pressure and heart rate were directly obtained from the physiological
573 recordings. Cardiac output was estimated as 1.61 fold the measurement of aortic blood flow.
574 This adjustment factor was obtained in a separate study in domestic chickens in which we

575 measured blood flow in the aorta and in both brachiocephalic arteries. Brachiocephalic flow
576 amounted to 32.6% of the total flow while coronary flow was estimated as 5.78% of the
577 total cardiac output [103]. These validation measurements are shown in Supplementary
578 Figure 5.
579
580 **Metabolic and thermoregulatory response to an aerobic challenge test**
581 For the aerobic challenge test both tinamou species and Red Junglefowl (or bantam
582 chickens in the case of the body temperature measurements) were moved to a larger room
583 (15-30 m^2) where they were chased by a researcher and forced to carry out three short
584 flapping flights and be on the run for a period of three minutes. After this time the animals
585 were placed on their backs to trigger righting reflexes consecutively until the animals went
586 into tonic immobility. When this occurred the challenge was concluded and we proceeded
587 with the post-challenge measurements. The short duration of the challenge was dictated by
588 the low stamina shown by tinamous in previous pilot runs, which correspond well to the
589 escape behaviour described in field studies [45]. In general, tinamous prefer running to
590 flying and only take off when pressed for it [45]. After the challenge both species of
591 tinamous appeared unequivocally exhausted and displayed fast gular fluttering, but not Red
592 Junglefowl or bantam chickens.
593 The aerobic challenge test was used in three procedures carried out separately: 1)
594 blood lactate determination, 2) oxygen consumption measurements and 3) body
595 temperature monitoring.
596 Blood lactate was measured before and immediately after the challenge test taking a
597 small blood sample from an ulnar vein punction. The blood sample was processed for
598 immediate determination of lactate concentration using a portable analyser (Lactate-Pro,

599 Arkray Inc, Kyoto, Japan). The lactate analyser has been previously validated for use with
600 bird blood [104].

601 Oxygen consumption was measured by open respirometry in a push-mode
602 configuration before and immediately after the aerobic challenge test previously described.
603 The animal was placed in a 6 liter air-tight chamber (18 cm diameter x 25 cm height)
604 equipped with two sets of tubing (Tygon R3603 3.2 x 4.8 mm) leading air in and out with a
605 controlled flow of 1200 ml min^{-1} (FOX II Analyzer, Sable Systems International, Las
606 Vegas, USA). To avoid dilution effects by the presence of water vapor, the air sample was
607 dried through a desiccator column (30 ml of indicating drierite, anhydrous calcium sulfate
608 mixed with cobalt chloride, W. A. Hammond Drierite company Ltd, Xenia, USA). The
609 FOX II Analyzer was connected to a laptop computer (Dell Latitude D600, Dell Inc.,
610 Round Rock, Texas, USA) via a serial connection and that data was stored using a custom
611 made data acquisition program (Lab View 8.6, National Instruments, Austin, Texas, USA).
612 Chamber flow (1200 ml min^{-1}) was set according to chamber volume and predicted VO_2
613 using published recommendations [105]. Oxygen consumption was calculated using
614 standard equations for the case when water vapor but not CO_2 is stripped from the gas
615 sample [106] as follows: V_{O2} = Flow x ($[O_2]$in-$[O_2]$out) / (1-0.2 x $[O_2]$out) where $[O_2]$in
616 and $[O_2]$out are the concentrations of oxygen entering and exiting the chamber respectively.

617 Body temperature was measured using a T-type thermocouple (RET-2, MLT1403,
618 ADInstruments-Europe, Oxford, UK) inserted in the cloaca an average length of 55 mm
619 and the leading wire was fixed with tape to the root of a tail feather. The thermocouple was
620 connected to a temperature pod (T-type Pod ML312, ADInstruments-Europe, Oxford, UK),
621 a Powerlab amplifier (Model 4/35 ADInstruments-Europe, Oxford, UK) and finally the
622 data was logged in a computer using LabChart 7Pro software (ADInstruments-Europe,

623 Oxford, UK). To account for the effect of circadian rhythms all measurements were carried
624 out between 10.00 and 15.00. A bird was taken from its cage without struggle in resting
625 conditions and placed in 6-L plastic containers (18 cm diameter x 25 cm height) for a
626 period of 3 h (pre-challenge baseline). After the aerobic challenge the animal was re-
627 instrumented with the cloacal probe and immediately returned to the holding container for
628 subsequent measurements (post-challenge, 2h). Measurements were carried out at two
629 different ambient temperatures, at 25°C (range 23.2-27°C) in an incubator (Yonar
630 Incubadoras, Buenos Aires, Argentina) or 5°C (range 3.8-5.6°C) in a refrigerator (FR093R,
631 Daewoo Electronics Corp., Seoul, South Korea). Ambient temperatures in the enclosures at
632 the time the measurements were made varied between 10-15°C.
633
634 **Gene expression**
635 Myocardial tissue from the three species was obtained post-mortem and preserved in
636 RNA*later*® Tissue Collection: RNA stabilization solution (Applied Biosystems, Thermo
637 Fisher Scientific, Walldorf, Germany) for 48 h at 4°C and later at -80°C prior to tissue
638 processing. Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific,
639 Walldorf, Germany) and reverse transcribed into cDNA using Revert Aid H Minus First
640 strand cDNA synthesis kit with Oligo(dT)$_{18}$ primers (Fermentas, Burlington, ON, Canada).
641 Quantitative real-time PCR was carried out using the Roche Light-cycler 480 (Roche
642 Applied Science, Roche Diagnostics, Basel, Switzerland) and Maxima SYBR Green qPCR
643 master mix (Fermentas, Burlington, ON, Canada). Levels of *ERK2*, *p38*, *JNK1* and
644 *PIK3CA* transcripts were normalized to the expression of *TBP*, *ACTB* and *GAPDH* using
645 the ΔCt-method. Gene nomenclature and specific primers are provided in Supplementary
646 Table 1.

647

648 **Statistical Analysis**

649 All results are presented as average with standard deviations (SD) following the
650 guidelines of the American Physiological Society for reporting statistics [107]. Statistical
651 analysis was carried out using general linear models followed by posthoc Tukey tests
652 (Minitab v.17, MiniTab Inc, State College, PA, USA) or permutation tests (StatBoss
653 permutation tester, M.J.Lew, Department of Pharmacology, The University of Melbourne,
654 [108]). Permutation tests are adequate and more robust than parametric tests to compare
655 differences between groups [108-110].

656 Power regression analysis on cardiac growth for the different species was carried out
657 after double logarithm transformation and Model II regression analysis (orthogonal
658 regression) in Minitab (v.17, MiniTab Inc, State College, PA, USA). Model II regression
659 was preferred over Model I regression because both variables (body mass and ventricular
660 mass) are obtained experimentally and include random measurement error [111, 112].

661 Specific details on statistical procedures are detailed in each figure legend.

662

663 **ACKNOWLEDGEMENTS**

664 Many people contributed to make this study possible: Ernesto Hug, Fernando Gianini,
665 Marcos Olivares, Oscar Cárdenas and Prof. Ekkehard Jordan took us to the Bolivian
666 Highlands in fruitful and rewarding hunting trips. Carmen Guzmán was crucial in the
667 logistics for all the work at the Chilean Tinamou farm. Andreas Calais and Johan Jonsson
668 carried out metabolic measurements in the Chilean Tinamous hosted by Roberto Nespolo
669 and Juan Diego Gaitán-Espitia at the Instituto de Ciencias Ambientales y Evolutivas,
670 Universidad Austral de Chile in Valdivia. Hanna Österman conducted the gene expression
671 study. Emma Backlund assisted in the echocardiography study in La Paz. Joaquim and
672 Anna Altimiras helped with the *in vivo* experiments on Red Junglefowl. Andrea Salazar
673 helped with animal care with tinamous and chickens. Finally, John Lees and Magnus
674 Elfwing provided fruitful criticisms to manuscript drafts.

675

676 **COMPETING INTERESTS**

677 The authors declare no financial or non-financial competing interests.

REFERENCES

1. Feduccia A. Bird origins anew. The Auk. 2013;130(1):1-12.
2. Feduccia A. Fantasy vs. reality: a critique of Smith *et al*.'s bird origins. Open Ornithology Journal. 2016;9:14-38.
3. Smith NA, Chiappe LM, Clarke JA, Edwards SV, Nesbitt SJ, Norell MA, et al. Rhetoric vs. reality: a commentary on "Bird Origins Anew" by A.Feduccia. The Auk. 2015;132:467-80. doi: 10.1642/AUK-14-203.1.
4. Dececchi TA, Larsson HCE, Habib MB. The wings before the bird: an evaluation of flapping-based locomotory hypotheses in bird antecedents. PeerJ. 2016;4:e2159. doi: 10.7717/peerj.2159.
5. Padian K, Chiappe LM. The origin and early evolution of birds. BiolRev. 1998;73:1-42.
6. Xu X, Zhou Z, Dudley R, Mackem S, Chuong C-M, Erickson GM, et al. An integrative approach to understanding bird origins. Science. 2014;346:1253293. doi: 10.1126/science.1253293.
7. Brusatte SL. A mesozoic aviary. Science. 2017;355(6327):792-4.
8. Allen V, Bates KT, Li Z, Hutchinson JR. Linking the evolution of body shape and locomotor biomechanics in bird-line archosaurs. Nature. 2013;497(7447):104-7. doi: 10.1038/nature12059. PubMed PMID: 23615616.
9. Evangelista D, Cam S, Huynh T, Kwong A, Mehrabani H, Tse K, et al. Shifts in stability and control effectiveness during evolution of Paraves support aerial maneuvering hypotheses for flight origins. PeerJ. 2014;2:e632. doi: 10.7717/peerj.632. PubMed PMID: 25337460; PubMed Central PMCID: PMCPMC4203027.
10. Feo TJ, Field DJ, Prum RO. Barb geometry of asymmetrical feathers reveals a transitional morphology in the evolution of avian flight. Proceedings of the Royal Society B: Biological Sciences. 2015;282(1803):20142864. doi: 10.1098/rspb.2014.2864. PubMed PMID: 25673687; PubMed Central PMCID: PMCPMC4345455.
11. Ruben JA. Reptilian physiology and the flight capacity of *Archaeopteryx*. Evolution. 1991;45(1):1-17.
12. Bishop CM. Circulatory variables and the flight performance of birds. JexpBiol. 2005;208:1695-708.
13. Dial KP. Wing-assisted incline running and the evolution of flight. Science. 2003;299(5605):402-4. doi: 10.1126/science.1078237. PubMed PMID: 12532020.
14. Heers AM, Baier DB, Jackson BE, Dial KP. Flapping before Flight: High Resolution, Three-Dimensional Skeletal Kinematics of Wings and Legs during Avian Development. PLoS One. 2016;11(4):e0153446. doi: 10.1371/journal.pone.0153446. PubMed PMID: 27100994; PubMed Central PMCID: PMCPMC4872793.
15. Hutchinson JR, Allen V. The evolutionary continuum of limb function from early theropods to birds. Naturwissen. 2009;96(4):423-48. doi: 10.1007/s00114-008-0488-3. PubMed PMID: 19107456.
16. Bishop CM. The maximum oxygen consumption and aerobic scope of birds and mammals: getting to the heart of the matter. ProcRoySocLondonSerB. 1999;266:2275-81.
17. De La Riboisiere J. Recherches organométriques en fonction du régime alimentaire sur les oiseaux [Docteur]. Paris: Université de Paris; 1910.
18. Dorst J. Poids relatif du coeur chez quelques oiseaux des hautes Andes du Perou. L'oiseau et la revue française d'ornithologie. 1972;42(1):66-73.

19. Hartman FA. Locomotor mechanisms of birds. Smithsonian Miscellaneous Collections. 1961;143(1):1-91.
20. Livezey BC, Zusi RL. Higher-order phylogeny of modern birds (Theropoda, Aves: Neornithes) based on comparative anatomy. II. Analysis and discussion. ZoolJLinSoc. 2007;149:1-95.
21. Prum RO, Berv JS, Dornburg A, Field DJ, Townsend JP, Lemmon EM, et al. A comprehensive phylogeny of birds (Aves) using targeted next-generation DNA sequencing. Nature. 2015;526(7574):569-73. doi: 10.1038/nature15697 http://www.nature.com/nature/journal/v526/n7574/abs/nature15697.html#supplementary-information.
22. Sibley CG, Ahlqvist JE. Phylogeny and classification of birds: a study in molecular evolution. New Haven: Yale University Press; 1990.
23. Cabot J. Family Tinamidae (tinamous). In: del Hoyo J, Elliott A, Sargatal J, editors. Handbook of the birds of the world Vol1. Barcelona: Lynx Edicions/Birdlife International; 1992. p. 112-38.
24. Davies SJJF. Ratites and tinamous. Oxford: Oxford University Press; 2002.
25. Bishop CM. Heart mass and the maximum cardiac output of birds and mammals: implications for estimating the maximum aerobic power input of flying animals. PhilTransRoySocLondonB. 1997;352:447-56.
26. Eme J, Gwalthney J, Blank JM, Owerkowicz T, Barron G, Hicks JW. Surgical removal of right-to-left cardiac shunt in the American alligator (*Alligator mississippiensis*) causes ventricular enlargement but does not alter apnoea or metabolism during diving. JexpBiol. 2009;212:3553-63.
27. Eme J, Gwalthney J, Owerkowicz T, Blank JM, Hicks JW. Turning crocodilian hearts into bird hearts: growth rates are similar for alligators with and without right-to-left cardiac shunt. JexpBiol. 2010;213:2673-80.
28. Nespolo RF, González-Lagos C, Solano-Iguaran JJ, Elfwing M, Garitano-Zavala A, Mañosa S, et al. The adaptive evolution of flight mode and aerobic power in birds: a phylogenetic test of the heart-size hypothesis. JexpBiol. 2017;submitted.
29. Grubb BR. Cardiac output and stroke volume in exercising ducks and pigeons. JApplPhysiol. 1982;53(1):207-11.
30. Grubb BR, Jorgensen DD, Conner M. Cardiovascular changes in the exercising emu. JexpBiol. 1983;104:193-201.
31. Jensen B, Elfwing M, Elsey RM, Wang T, Crossley DA, II. Coronary blood flow in the anesthetized American alligator (*Alligator mississippiensis*). Comparative Biochemistry and Physiology Part A. 2016;191:44-52.
32. Martínez-Lemus LA, Miller MW, Jeffrey JS, Odom TW. Echocardiographic evaluation of cardiac structure and function in broiler and Leghorn chickens. PoultSci. 1998;77:1045-50.
33. Seymour RS, Blaylock AJ. The Principle of Laplace and Scaling of Ventricular Wall Stress and Blood Pressure in Mammals and Birds. PhysiolBiochemZool. 2000;73(4):389-405.
34. Burton RR, Smith AH. Induction of cardiac hypertrophy and polycythemia in the developing chick at high altitude. FedProc. 1969;28(3):1170-7.
35. Withers PC, Forbes RB, Hedrick MS. Metabolic, water and thermal relations of the Chilean Tinamou. The Condor. 1987;89:424-6.

769 36. McNab BK. Energy conservation and the evolution of flightlessness in birds. The
770 American Naturalist. 1994;144(4):628-42.
771 37. McNab BK. Extreme measures. The ecological energetics of birds and mammals.
772 Chicago: The University of Chicago Press; 2012.
773 38. Viscor G, Fuster JF. Relationships between morphological parameters in birds with
774 different flying habits. CompBiochemPhysiolA. 1987;87(2):231-49.
775 39. Franklin CE, Davis BM, Peucker SKJ, Stephenson H, Mayer RE, Whittier J, et al.
776 Comparison of stress induced by manual restraint and immobilisation in the estuarine
777 crocodile, *Crocodylus porosus*. Journal of Experimental Zoology A. 2003;298A(2):86-92.
778 40. Gleeson TT. Post-exercise lactate metabolism: a comparative review of sites,
779 pathways, and regulation. AnnuRevPhysiol. 1996;58:565-81.
780 41. Gaesser GA, Brooks GA. Metabolic bases of excess post-exercise oxygen
781 consumption: a review. MedSciSportsExercise. 1984;16(1):29-43.
782 42. Baker EJ, Gleeson TT. EPOC and the energetics of brief locomotor activity in *Mus*
783 *domesticus*. Journal of Experimental Zoology A. 1998;280(2):114-20.
784 43. Gleeson TT, Hancock TV. Modeling the Metabolic Energetics of Brief and
785 Intermittent Locomotion in Lizards and Rodents. AmerZool. 2001;41(2):211-8. doi:
786 10.1093/icb/41.2.211.
787 44. Clarke A, Rothery P. Scaling of body temperature in mammals and birds. FuncEcol.
788 2008;22:58-67.
789 45. Pearson AK, Pearson OP. Natural history and breeding behavior of the tinamou,
790 *Nothoprocta ornata*. The Auk. 1955;72(2):113-27.
791 46. Heineke J, Molkentin JD. Regulation of cardiac hypertrophy by intracellular
792 signalling pathways. Nature Reviews Molecular Cell Biology. 2006;7:589-600.
793 47. Rose BA, Force T, Wang Y. Mitogen-activated protein kinase signaling in the heart:
794 angels versus demons in a heart-breaking tale. Physiol Rev. 2010;90(4):1507-46. doi:
795 10.1152/physrev.00054.2009. PubMed PMID: 20959622; PubMed Central PMCID:
796 PMCPMC3808831.
797 48. Liberatore CM, Yutzey KE. MAP kinase activation in avian cardiovascular
798 development. DevelopDynam. 2004;230:773-80.
799 49. Liang Q, Bueno OF, Wilkins BJ, Kuan CY, Xia Y, Molkentin JD. c-Jun N-terminal
800 kinases (JNK) antagonize cardiac growth through cross-talk with calcineurin–NFAT
801 signaling. The EMBO Journal. 2003;22(19):5079-89. doi: 10.1093/emboj/cdg474.
802 50. Braz JC, Bueno OF, Liang Q, Wilkins BJ, Dai Y-S, Parsons S, et al. Targeted
803 inhibition of p38 MAPK promotes hypertrophic cardiomyopathy through upregulation of
804 calcineurin-NFAT signaling. The Journal of Clinical Investigation. 2003;111(10):1475-86.
805 doi: 10.1172/JCI17295.
806 51. Cleland TP, Stoskopf MK, Schweitzer MH. Histological, chemical, and
807 morphological reexamination of the "heart" of a small Late Cretaceous Thescelosaurus.
808 Naturwissen. 2011;98(3):203-11. doi: 10.1007/s00114-010-0760-1. PubMed PMID:
809 21279321.
810 52. Schweitzer MH. Soft tissue preservation in terrestrial mesozoic vertebrates. Annual
811 Review of Earth and Planetary Sciences. 2011;39:187-216. doi: 10.1146/annurev-earth-
812 040610-133502.
813 53. Feduccia A, Czerkas SA. Testing the neoflightless hypothesis: propatagium reveals
814 flying ancestry of oviraptorosaurs. Journal of Ornithology. 2015;156(4):1067-74. doi:
815 10.1007/s10336-015-1190-9.

816 54. Wang M, Zhou Z. The evolution of birds with implications from new fossil evidences.
817 In: Maina JN, editor. The biology of the avian respiratory system Evolution, development,
818 structure and function: Springer; 2017. p. 1-26.
819 55. Sanz JL, Chiappe LM, Pérez-Moreno BP, Buscalioni AD, Moratalla JJ, Ortega F, et
820 al. An Early Cretaceous bird from Spain and its implications for the evolution of avian
821 flight. Nature. 1996;382:442-5.
822 56. Navalón G, Marugán-Lobón J, Chiappe LM, Sanz JL, Buscalioni AD. Soft-tissue and
823 dermal arrangement in the wing of an Early Cretaceous bird: implications for the evolution
824 of avian flight. Scientific Reports. 2015;5:14864. doi: 10.1038/srep14864.
825 57. Wang M, Zheng X, O'Connor JK, Lloyd GT, Wang X, Wang Y, et al. The oldest
826 record of ornithuromorpha from the early cretaceous of China. Nature Communications.
827 2015;6:6987. doi: 10.1038/ncomms7987.
828 58. Earls KD. Kinematics and mechanics of ground take-off in the starling *Sturnis*
829 *vulgaris* and the quail *Coturnix coturnix*. JexpBiol. 2000;203:725-39.
830 59. Schaller NU, Herkner B, Villa R, Aerts P. The intertarsal joint of the ostrich (*Struthio*
831 *camelus*): Anatomical examination and function of passive structures in locomotion. JAnat.
832 2009;214:830-47.
833 60. Bundle MW, Hoppeler H, Vock R, Tester JM, Weyand PG. High metabolic rates in
834 running birds. Nature. 1999;397(6714):31-2.
835 61. Bourdon E, De Ricqles AJ, Cubo J. A new Transantarctic relationship: morphological
836 evidence for a Rheidae-Dromaiidae-Casuariidae clade (Aves, Palaeognathae, Ratitae).
837 ZoolJLinSoc. 2009;156:641-63.
838 62. Mayr G, Clarke J. The deep divergences of neornithine birds: a phylogenetic analysis
839 of morphological characters. Cladistics. 2003;19:527-53.
840 63. Worthy TH, Scofield RP. Twenty-first century advances in knowledge of the biology
841 of moa (Aves: Dinornithiformes): a new morphological analysis and moa diagnoses revised.
842 NZJZool. 2012;39(2):87-153.
843 64. Nesbitt SJ, Clarke JA. The anatomy and taxonomy of the exquisitely preserved Green
844 River formation (early Eocene) Lithornithids (Aves) and the relationships of Lithornithidae.
845 BullAmMusNatHist. 2016;406:1-91.
846 65. Baker AJ, Haddrath O, McPherson JD, Cloutier A. Genomic support for a moa-
847 tinamou clade and adaptive morphological convergence in flightless Ratites. MolBiolEvol.
848 2014;31(7):1686-96. doi: 10.1093/molbev/msu153.
849 66. Hackett SJ, Kimball RT, Reddy S, Bowie RCK, Braun EL, Braun MJ, et al. A
850 phylogenomic study of birds reveals their evolutionary history. Science. 2008;320:1763-8.
851 67. Haddrath O, Baker AJ. Multiple nuclear genes and retroposons support vicariance
852 and dispersal of the palaeognaths, and an Early Cretaceous origin of modern birds.
853 Proceedings of the Royal Society B: Biological Sciences. 2012;279:4617-25. doi:
854 10.1098/rspb.2012.1630.
855 68. Harshman J, Braun EL, Braun MJ, Huddleston CJ, Bowie RCK, Chojnowski JL, et al.
856 Phylogenomic evidence for multiple losses of flight in ratite birds. ProcNatAcadSciUSA.
857 2008;105(36):13462-7.
858 69. Mitchell KJ, Llamas B, Soubrier J, Rawlence NJ, Worthy TH, Wood J, et al. Ancient
859 DNA reveals elephant birds and kiwi are sister taxa and clarifies ratite bird evolution.
860 Science. 2014;344:898-900.

861 70. Phillips MJ, Gibb GC, Crimp EA, Penny D. Tinamous and moa flock together:
862 mitochondrial genome sequence analysis reveals independent losses of flight among ratites.
863 SystBiol. 2010;59(1):90-107.
864 71. Smith JV, Braun EL, Kimball RT. Ratite non-monophyly: independent evidence from
865 40 novel loci. SystBiol. 2012;62(1):35-49. doi: 10.1093/sysbio/sys067.
866 72. Yonezawa T, Segawa T, Mori H, Campos PF, Hongoh Y, Endo H, et al.
867 Phylogenomics and Morphology of Extinct Paleognaths Reveal the Origin and Evolution of
868 the Ratites. Current Biology. 2017;27(1):68-77. doi:
869 http://dx.doi.org/10.1016/j.cub.2016.10.029.
870 73. Jarvis ED, Mirarab S, Aberer AJ, Li B, Houde P, Li C, et al. Whole-genome analyses
871 resolve early branches in the tree of life of modern birds. Science. 2014;346(6215):1320-31.
872 doi: 10.1126/science.1253451.
873 74. Mayr G. Avian Evolution. The fossil record of birds and its paleobiological
874 significance. Benton MJ, editor: Wiley Blackwell; 2017.
875 75. Kurochkin EN, Duyke GJ, Karhu AA. A new presbyornithid bird (Aves,
876 Anseriformes) from the Late Cretaceous of Southern Mongolia. AmMusNovitates. 2002:1-
877 11.
878 76. Clarke JA, Tambussi CP, Noriega JI, Erickson GM, Ketcham RA. Definitive fossil
879 evidence for the extant avian radiation in the Cretaceous. Nature. 2005;433:305-8.
880 77. Clarke JA. Morphology, phylogenetic, taxonomy, and systematics of *Ichthyornis* and
881 *Apatornis* (Avialae: Ornithurae). BullAmMusNatHist. 2004;286:1-179.
882 78. Agnolin FL. Unexpected diversity of ratites (Aves, Palaeognathae), in the early
883 Cenozoic of South America: palaeobiogeographical implications. Alcheringa: An
884 Australasian Journal of Palaeontology. 2016. doi: 10.1080/03115518.2016.1184898.
885 79. Mayr G. Paleogene fossil birds. Berlin: Springer; 2009.
886 80. Bertelli S, Chiappe LM, Mayr G. Phylogenetic interrelationships of living and extinct
887 Tinamidae, volant palaeognathous birds from the New World. ZoolJLinSoc.
888 2014;172(1):145-84.
889 81. Houde P, Olson SL. Paleognathous carinate birds from the early Tertiary of North
890 America. Science. 1981;214:1236-7.
891 82. Leonard L, Dyke GJ, Van Tuinen M. A new specimen of the fossil palaeognath
892 *Lithornis* from the Lower Eocene of Denmark. AmMusNovitates. 2005;3491.
893 83. Grellet-Tinner G, Dyke GJ. The eggshell of the Eocene bird *Lithornis*. Acta
894 Palaeontologica Polonica. 2005;50(4):831-5.
895 84. Grealy A, Phillips M, Miller G, Gilbert MTP, Rouillard J-M, Lambert D, et al.
896 Eggshell palaeogenomics: Palaeognath evolutionary history revealed through ancient
897 nuclear and mitochondrial DNA from Madagascan elephant bird (Aepyornis sp.) eggshell.
898 Molecular Phylogenetics and Evolution. 2017;109:151-63. doi:
899 https://doi.org/10.1016/j.ympev.2017.01.005.
900 85. Ezcurra MD, Agnolín FL. A new global palaeobiogeographical model for the late
901 Mesozoic and early Tertiary. SystBiol. 2012;61(4):553-66. doi: 10.5061/dryad.d47h94c9).
902 86. Gorscak E, O'Connor PM. Time-calibrated models support congruency between
903 Cretaceous continental rifting and titanosaurian evolutionary history. Biology Letters.
904 2016;12(4). doi: 10.1098/rsbl.2015.1047. PubMed PMID: 27048465; PubMed Central
905 PMCID: PMCPMC4881341.

87. Hoernle K, van den Bogaard P, Werner R, Lissinna B, Hauff F, Alvarado G, et al. Missing history (16-71 Ma) of the Galápagos hotspot: implications for the tectonic and biological evolution of the Americas. Geology. 2002;30(9):795-8.
88. Stidham TA, Lofgren D, Farke AA, Paik M, Choi R. A lithornitid (Aves: Palaeognathae) from the Paleocene (Tiffanian) of southern California. PaleoBios. 2014;31(1):1-7.
89. Schütz KE, Forkman B, Jensen P. Domestication effects on foraging strategy, social behaviour and different fear responses: a comparison between the red junglefowl (*Gallus gallus*) and a modern layer strain. ApplAnimBehavSci. 2001;74:1-14.
90. Bezuidenhout AJ. The valva atrioventricularis dextra of the avian heart. Anatomia Histologia Embryologia. 1983;12:104-8.
91. Lu Y, James TN, Bootsma M, Terasaki F. Histological organization of the right and left atrioventricular valves of the chicken heart and their relationship to the atrioventricular Purkinje ring and the middle bundle branch. AnatRec. 1993;235:74-86.
92. Österman H, Lindgren I, Lindström T, Altimiras J. Chronic hypoxia during development does not trigger pathologic remodeling of the chicken embryonic heart but reduces cardiomyocyte number. American Journal of Physiology - Regulatory Integrative and Comparative Physiology. 2015;309:R1204-R14. doi: 10.1152/ajpregu.00499.2014.
93. Grubb BR. Allometric relations of cardiovascular function in birds. AmerJPhysiolHeart CircPhysiol. 1983;245:H567-H72.
94. Bagatto B, Crossley DA, II, Altimiras J, Elsey RM, Hicks JW. Physiological variability in yearling alligators: Clutch differences at rest and during activity. CompBiochemPhysiolA. 2012;162(1):44-50. doi: 10.1016/j.cbpa.2012.02.005.
95. Crossley D, Altimiras J. Effect of selection for commercially productive traits on the plasticity of cardiovascular regulation in chicken breeds during embryonic development. PoultSci. 2012;91:2628-36. doi: 10.3382/ps.2012-02344.
96. Crossley DA, II, Altimiras J. Cardiovascular development in embryos of the American alligator *Alligator mississippiensis*: effects of chronic and acute hypoxia. JexpBiol. 2005;208:31-9. doi: 10.1242/jeb.01355.
97. Crossley DA, II, Hicks JW, Altimiras J. Ontogeny of baroreflex regulation in the American alligator *Alligator mississippiensis*. JexpBiol. 2003;206:2895-902. doi: 10.1242/jeb.00486.
98. Ingeström E. Phenotypic plasticity of the heart and skeletal muscles in cold acclimated Red Junglefowls (*Gallus gallus*) [Bachelor Thesis LiTH-IFM-Ex-15/3021-SE]. Linköping: University of Linköping; 2015.
99. Lindgren I, Altimiras J. Sensitivity of organ growth to chronically low oxygen levels during incubation in Red Junglefowl and domesticated chicken breeds. PoultSci. 2011;90(1):126-35. doi: 10.3382/ps.2010-00996.
100. Lindgren I, Altimiras J. Prenatal hypoxia programs changes in β-adrenergic signaling and postnatal cardiac contractile dysfunction. AmerJPhysiolHeart CircPhysiol. 2013;305:R1093-R101. doi: 10.1152/ajpregu.00320.2013.
101. Lindholm C, Calais A, Jönsson J, Yngwe N, Berndtson E, Hult E, et al. Slow and steady wins the race? No signs of reduced welfare in smaller broiler breeder hens at four weeks of age. Animal Welfare. 2015;24:447-54. doi: 10.7120/09627286.24.4.447.
102. Jones RB. The tonic immobility reaction of the domestic fowl: a review. World's Poultry Science Journal. 1986;42(1):82-96. doi: 10.1079/WPS19860008.

952 103. Boelkins JN, Mueller WJ, Hall KL. Cardiac output distributionin the laying hen
953 during shell formation. CompBiochemPhysiolA. 1973;46:735-43.
954 104. Lindholm C, Altimiras J. Point-of-care devices for physiological measurements in
955 field conditions. A smorgasbord of instruments and validation procedures. Comparative
956 Biochemistry and Physiology Part A- Molecular & Integrative Physiology. 2016;202:99-
957 111. doi: 10.1016/j.cbpa.2016.04.009.
958 105. McNab BK. The relationship among flow rate, chamber volume and calculated rate
959 of metabolism in vertebrate respirometry. CompBiochemPhysiolA. 2006;145:287-94.
960 106. Lighton JRB. Measuring metabolic rates. A manual for scientists. New York: Oxford
961 University Press; 2008.
962 107. Curran-Everett D, Benos DJ. Guidelines for reporting statistics in journals published
963 by the Americal Physiological Society. AmJPhysiol. 2004;287:R247-R9.
964 108. Ludbrook J, Dudley H. Why permutation tests are superior to t and F tests in
965 biomedical research. The American Statistician. 1998;52(2):127-32.
966 109. Drummond GB, Vowler SL. Different tests for a difference: how do we do research?
967 ExpPhysiol. 2012;97(2):171-4. doi: 10.1113/expphysiol.2011.063636.
968 110. Lew MJ. On contemporaneous controls, unlikely outcomes, boxes and replacing the
969 "Student": Good statistical practice in pharmacology, problem 3. BritJPharmacol.
970 2008;155:797-803.
971 111. Ludbrook J. Linear regression analysis for comparing two measurers or methods of
972 measurement: But which regression? ClinExpPharmacolPhysiol. 2010;37:692-9.
973 112. Ludbrook J. A primer for biomedical scientists on how to execute Model II linear
974 regression analysis. ClinExpPharmacolPhysiol. 2012;39:329-35.

Table 1. Relative body mass in all tinamou species recorded to date. Altitudinal and latitudinal distribution for each species were obtained from BirdLife International and NatureServe (2014) Bird Species Distribution Maps of the World. The IUCN Red List of Threatened Species. Version 2016-2. (http://maps.iucnredlist.org/index.html).

Species	Common name	Body mass (g)	%VM:BM	Altitudinal distribution	Latitudinal distribution
Subfamily Tinaminae (forest dwelling tinamous)					
Nothocercus bonapartei	Highland Tinamou	885.2	0.194[1]	500-2500 m	11°02'N – 05°21'S
Tinamus major	Great Tinamou	1169.4	0.147[1]	0-1500 m	18°55'N – 18°04'S
		1140.0	0.172[5]		
Crypturellus soui	Little Tinamou	233.0	0.194[1]	0-2000 m	19°07'N – 22°30'S
Crypturellus undulatus	Undulated Tinamou	537.5	0.187[5]	0-900 m	08°24'N – 27°47'S
Crypturellus parvirostris	Small-billed Tinamou	136.8	0.341[4]	0-1200 m	00°23'S – 28°36'S
Crypturellus tataupa	Tataupa Tinamou	275.0	0.192[5]	0-1400 m	02°20'S – 31°35'S
Subfamily Rhynchotinae (open fields dwelling tinamous)					
Rhynchotus rufescens	Red-winged Tinamou	821.7	0.198[3]	0-2500 m	03°26'S – 41°10'S
		796.6	0.190[5]		
Nothoprocta ornata	Ornate Tinamou	505.0	0.235[4]	2500-4800 m	07°40'S – 30°00'S
		500.0	0.224[5]		
Nothoprocta perdicaria	Chilean Tinamou	395.5	0.284[4]	400-2000 m	28°24'S – 41°46'S
Nothoprocta pentlandii	Andean Tinamou	258.0	0.298[5]	1500-4000 m	02°50'S – 36°54'S
Nothura boraquira	White-bellied Nothura	310.0	0.178[5]	0-500 m	03°12'S – 22°43'S
Nothura darwinii	Darwin's Nothura	251.4	0.280[4]	1000–4300 m	09°24'S – 44°29'S
		197.0	0.321[5]		
Nothura maculosa	Spotted Nothura	275.0	0.265[2]	0–2300 m	05°06'S – 44°18'S

[1] From Hartman, F.A., 1961. Locomotor mechanisms of birds. Smithsonian Miscellaneous Collections 143, 1-91.
[2] From Dorst, J., 1972. Poids relatif du coeur chez quelques oiseaux des hautes Andes du Perou. L'oiseau et la revue française d'ornithologie 42, 66-73.
[3] From De La Riboisiere, J., 1910. Recherches organométriques en fonction du régime alimentaire sur les oiseaux, Faculté des Sciences. Université de Paris, Paris.
[4] Own data from fresh specimens
[5] Own data from specimens preserved in ethanol

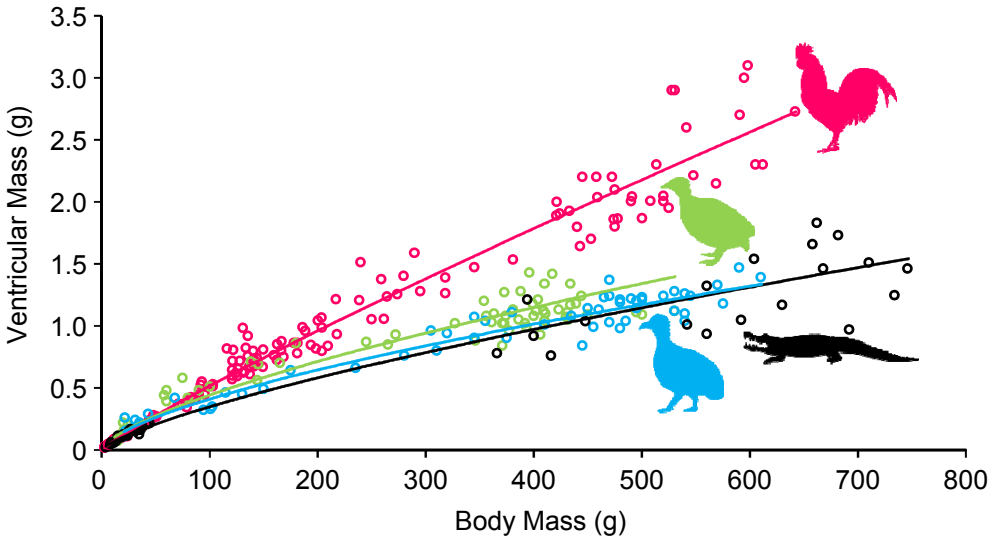


Figure 1

Cardiac growth from embryonic age to juvenile or adult age in Red Junglefowl (*Gallus gallus*) in red, Chilean Tinamou (*Nothoprocta perdicaria*) in green, Ornate Tinamou (*Nothoprocta ornata*) in blue and American alligator (*Alligator mississippiensis*) in black. Power regression lines for each species were obtained after logarithmic transformation and Model II analysis (orthogonal regression) in Minitab 17. The power regression equation are as follows: *Gg*: VM = 0.0085 $BM^{0.892}$; *Np*: VM = 0.0186 $BM^{0.688}$; *No*: VM = 0.0207 $BM^{0.649}$; *Am*: VM = 0.0117 $BM^{0.737}$.

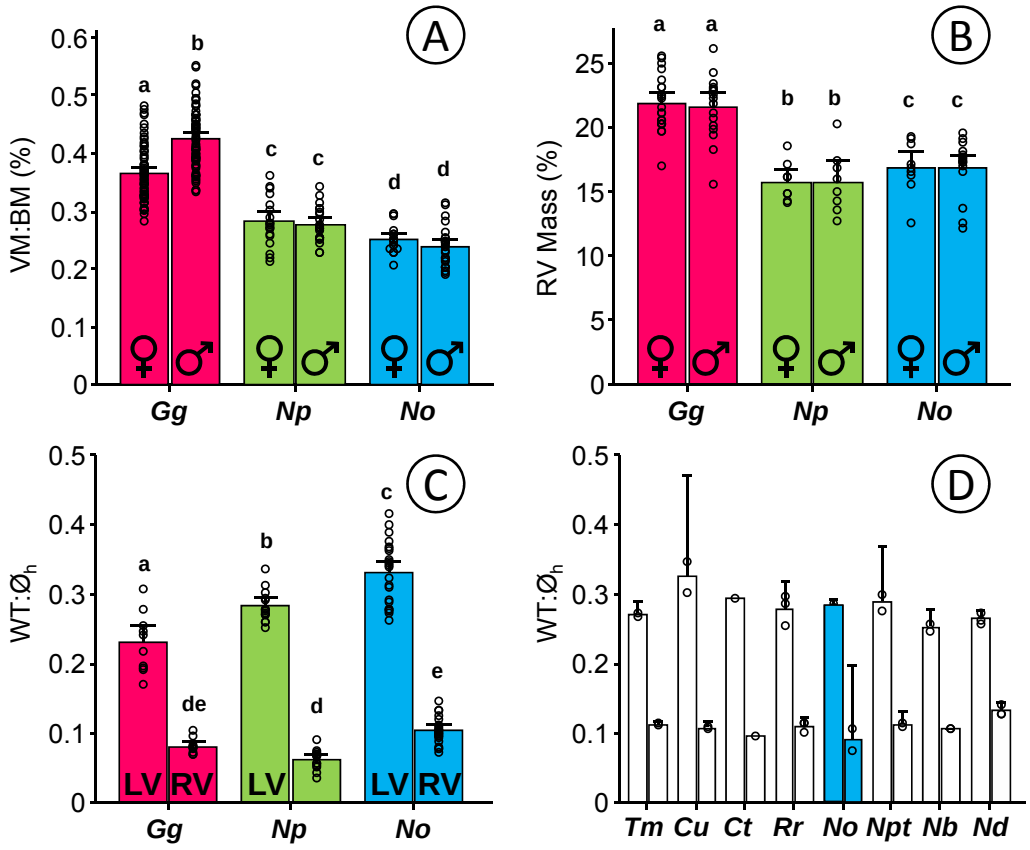


Figure 2

Comparative morphometry of the heart in adult specimens of Red Junglefowl (*Gallus gallus*, *Gg*), Chilean Tinamou (*Nothoprocta perdicaria*, *Np*) and Ornate Tinamou (*Nothoprocta ornata*, *No*). A) Relative ventricular mass in males and females shown as the percentage of ventricular mass to body mass (VM:BM). B) Mass of the right ventricle (RV) in males and females shown as the percentage of RV to VM. C) Left and right ventricular wall thickness normalized to the diameter of the heart (WT:$Ø_h$) obtained from the ventricular section showing the attachment of an incipient right atrioventricular valve to the right ventricular free wall (see Material and Methods for details). D) Normalized left and right ventricular wall thickness obtained as in panel C from ethanol preserved specimens of other tinamou species kept at the Colección Boliviana de Fauna at the Universidad Mayor de San Andrés in La Paz, Bolivia. Species nomenclature as in Table 1: *Tm* – *Tinamus major* (N=2), *Cu* – *Crypturellus undulatus* (N=2), *Ct* – *Crypturellus tataupa* (N=1), *Rr* – *Rhynchotus rufescens* (N=3), *No* – *Nothoprocta ornata* (N=2), *Npt* – *Nothoprocta pentlandii* (N=2), *Nb* – *Nothura boraquira* (N=2), *Nd* – *Nothura darwinii* (N=3). All data presented as mean and 95% confidence intervals with individual data points shown. N values as follows (in order from left to right in the different panels): A – 68,70,19,21,19,26; B – 21,17,8,8,9,15; C – 10,10,14,14,22,22. For statistical analysis for panels ABC we used general linear modeling (GLM) considering species and gender (AB) or species and ventricle (C) as factors followed by Tukey posthoc test with a customary fiduciary significant level of $p<0.05$ (shown as dissimilar letters) in Minitab 17. No statistical analysis was performed for panel D.

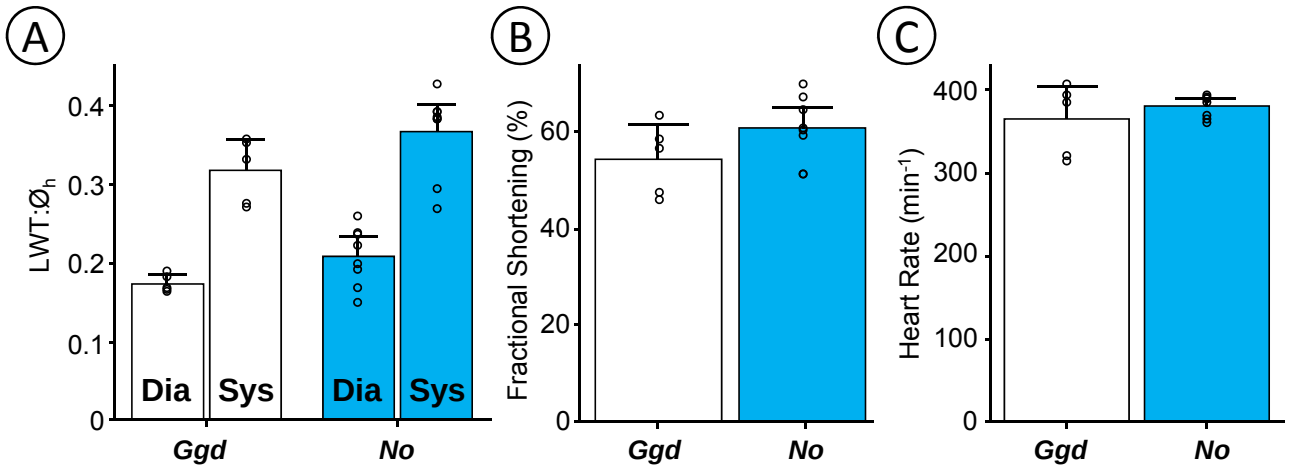


Figure 3

Functional echocardiographic measurements of the heart in conscious birds placed in supine position under tonic immobility. Bantam chickens (*Gallus gallus domesticus*, *Ggd*) were used for comparison with the Ornate Tinamou (*Nothoprocta ornata*, *No*). A) Left ventricular wall thickness normalized to the diameter of the heart (LWT:$Ø_h$) from a parasternal echocardiographic plane in which the right ventricular free wall is incipient but without a visible right ventricular chamber (see Material and Methods for details). B) Fractional Shortening (%) of the cardiac muscle at the same plane. C) Heart rate estimated from the time between subsequent peak systolic events in M-mode echo. All data presented as mean and 95% confidence intervals with individual data points shown (N=5 for *Ggd* and N=8 for *No*). Due to small sample size and an assumed lack of normality and homocedasticity of the data, permutation tests were used to test for differences between species using StatBoss (see Material and Methods for further details). No significant differences were found.

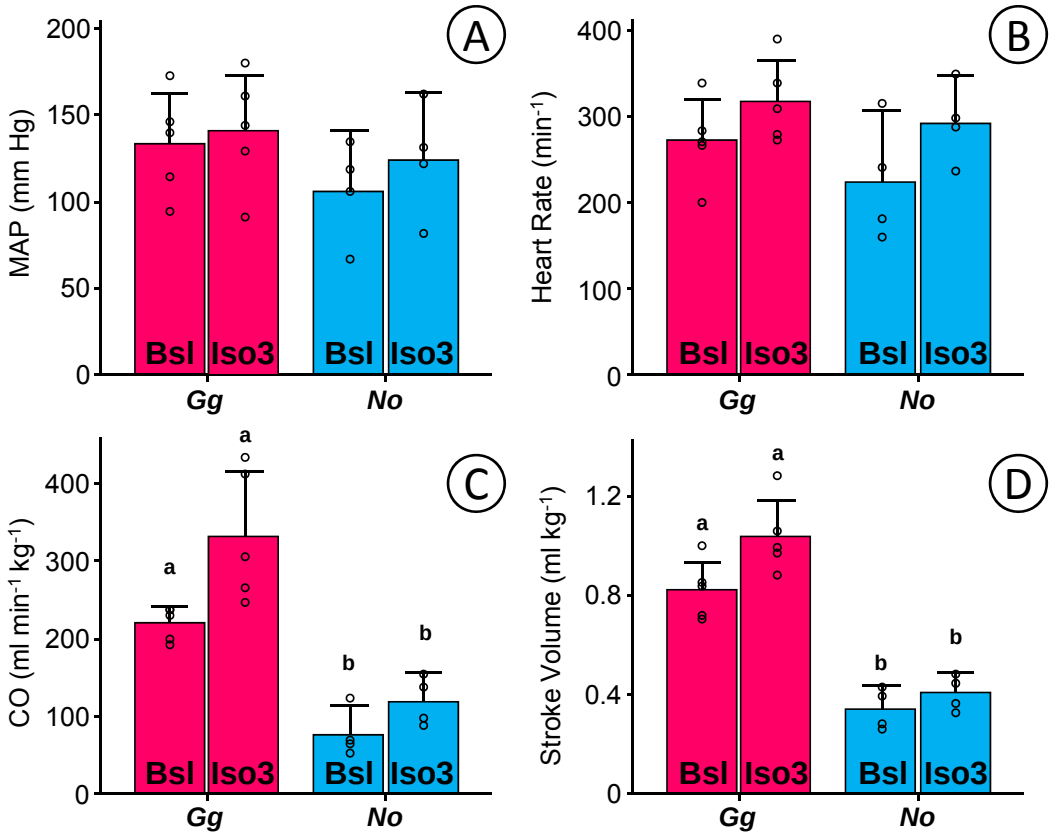


Figure 4

Functional measurements of cardiovascular variables in ketamine-xylazine anesthetized Red Junglefowl (*Gallus gallus*, *Gg*) and Ornate Tinamou (*Nothoprocta ornata, No*) before (Bsl – baseline) and after the administration of 3 ug kg^{-1} of isoproterenol (Iso3). A) Mean Arterial Pressure (MAP, mm Hg) measured from an intravascular catheter in the brachial artery. B) Heart Rate calculated from the instantaneous pressure trace. C) Mass specific total Cardiac Output (CO) estimated from a transit flow probe placed in the aortic arch after the splitting of the brachiocephalic arteries (see Material and Methods and Suppl.Figure 5 for details) and D) Stroke Volume calculated from the quotient between CO and heart rate. All data presented as mean and 95% confidence intervals with individual data points shown (N=5 for *Gg* and N=4 for *No*). Due to small sample size and an assumed lack of normality and homocedasticity of the data, paired permutation tests were used to test for differences between species and for the effect of isoproterenol treatment using StatBoss (see Material and Methods for further details). A customary fiduciary significant level of $p<0.05$ was used after compensation for multiple comparisons. Statistical differences between species but not due to treatment were seen only in panels C and D and are shown by dissimilar letters.

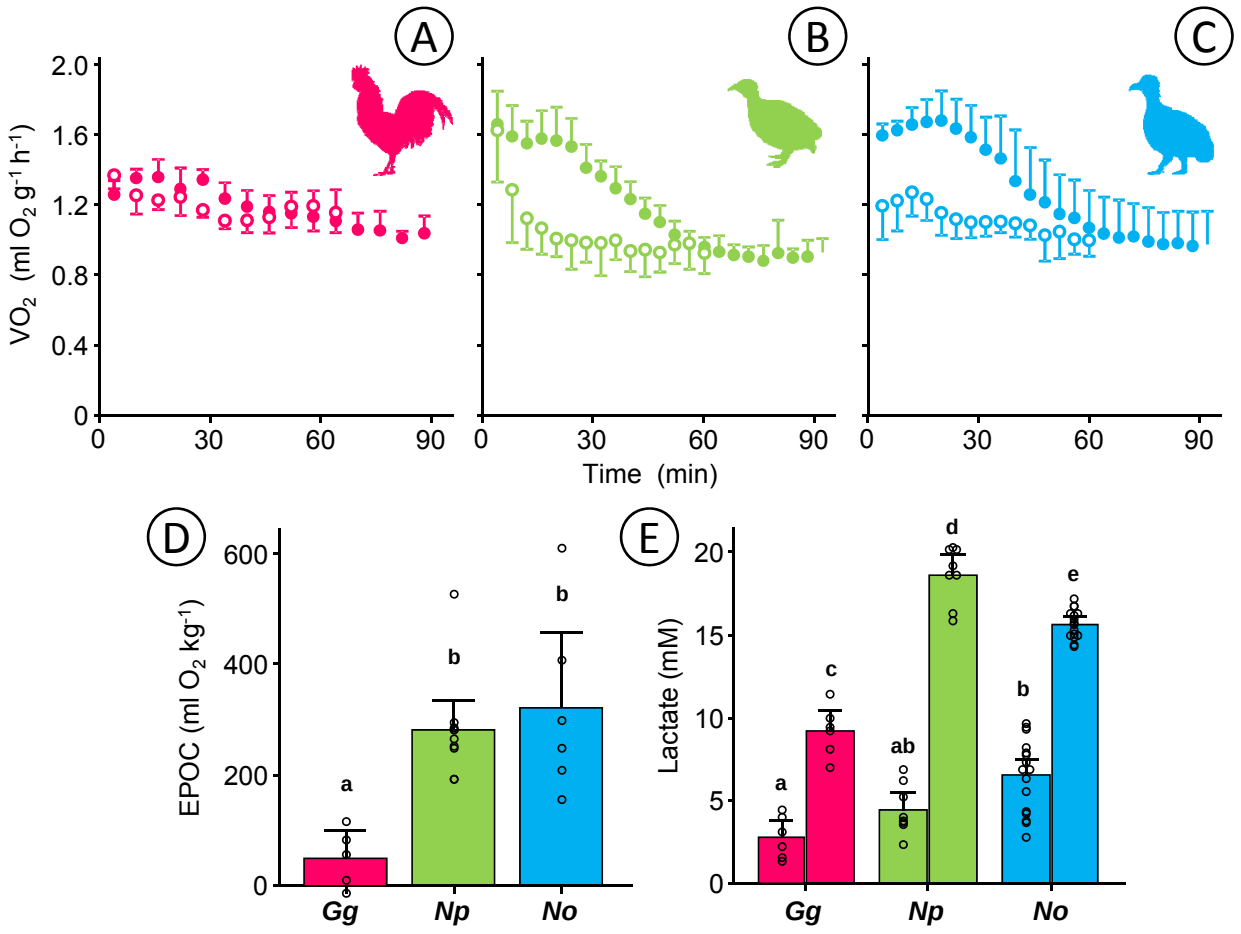


Figure 5

Metabolic measurements before and after a 3 min chase-and-exhaust protocol. ABC) Mass-specific oxygen consumption (VO_2) in adult individuals of A) Red Junglefowl (*Gallus gallus*, *Gg*, N=5), B) Chilean Tinamou (*Nothoprocta perdicaria*, *Np*, N=10) and C) Ornate Tinamou (*Nothoprocta ornata*, *No*, N=6). Open symbols show the data for the 60 min baseline measurements and closed symbols show the data for the 90 min following the chase protocol. D) Excess post-exercise oxygen consumption (EPOC) obtained by integrating the pre- and post-curves shown as ABC. E) Plasma lactate levels obtained using the same protocol in a separate group of individuals (*Gg* N=6, *Np* N=8, *No* N=16). Data in A-C presented as mean and standard deviations. Data in D-E presented as mean and 95% confidence intervals with individual data points shown. For statistical analysis we used general linear modeling (GLM) considering species (D) and species/treatment (E) as factors followed by Tukey posthoc test with a customary fiduciary significant level of $p<0.05$ (shown as dissimilar letters) in Minitab 17. No statistical analysis was performed for panels ABC because the integrated response is considered in panel D.

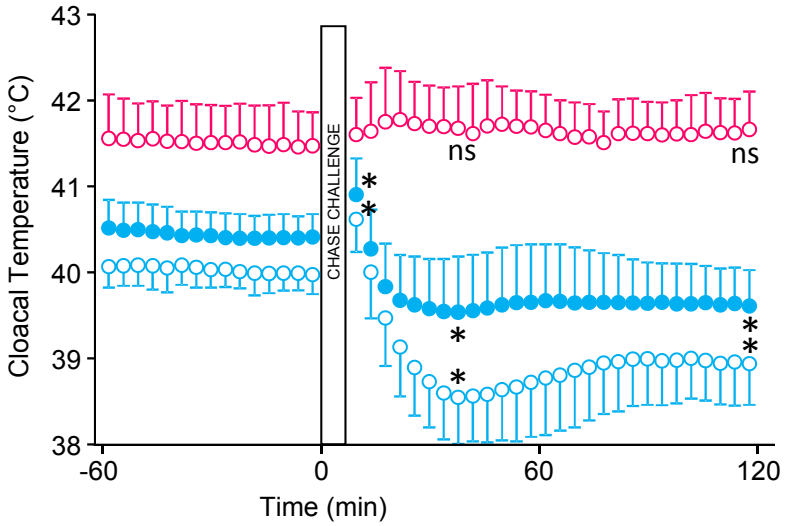


Figure 6

Cloacal temperature before and after a 3 min chase-and-exhaust protocol in Ornate Tinamou kept at an ambient temperature of 4°C (open blue symbols) and 25°C (closed blue symbols). The chase-and-exhaust protocol was carried out after a baseline measurement lasting 1 h. For comparison, data on cloacal temperature in bantam chickens kept at 4°C (open red symbols) that underwent the same protocol are shown. Data from chickens at 25°C did not differ substantially and is shown in Suppl.Fig.2. All data presented as mean and standard deviations (N=6 for both species). Paired permutation tests were used to compare baseline temperatures preceding the chase-and-exhaust protocols (120 min) with the temperatures 30 min after the test. Significant differences were observed only for the Ornate Tinamou (p=0.03 in both cases) and are shown by "*" in the graph. "ns" indicate no significant difference.

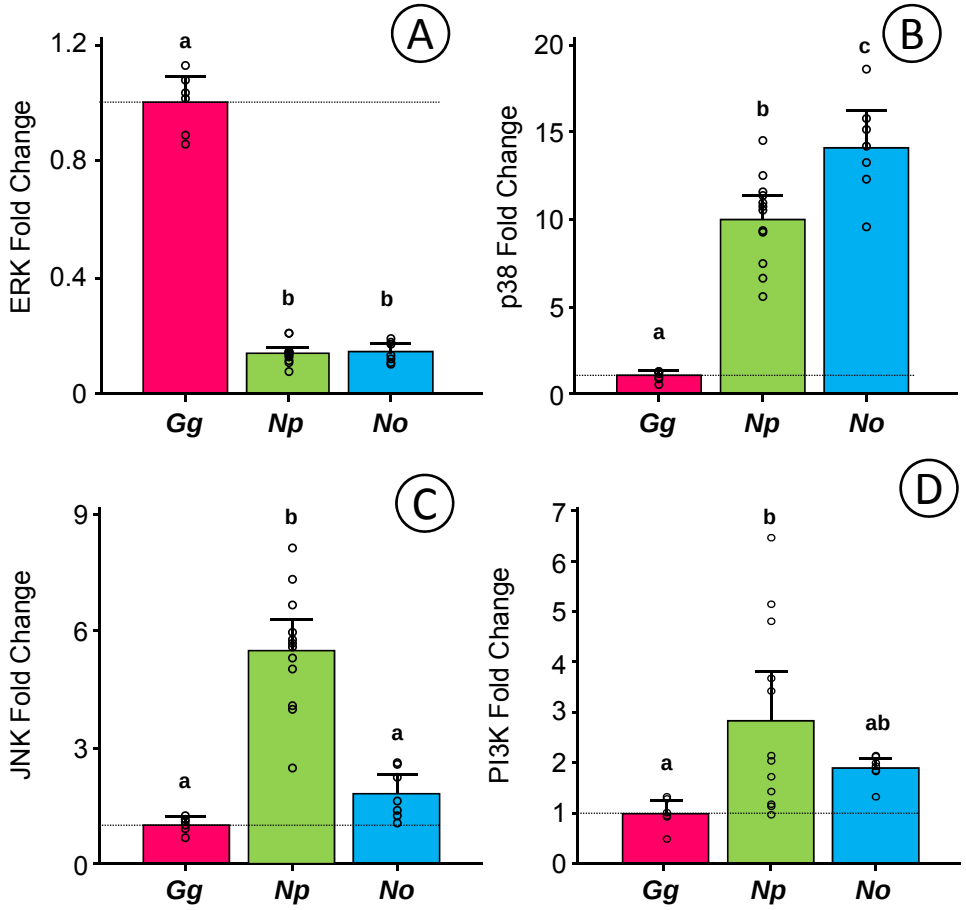


Figure 7

Relative expression of the three main MAP kinase genes: ERK (A), p38 (B) and Jnk (C) and PI3K (D) in Red Junglefowl (*Gallus gallus*, *Gg*, N=6), Chilean Tinamou (*Nothoprocta perdicaria*, *Np*, N=12) and Ornate Tinamou (*Nothoprocta ornata*, *No*, N=7). All values calculated in relation to the expression in Red Junglefowl after normalization against three housekeeping genes: GAPDH, β-actin and TBP. Dotted line indicates the reference expression level for Red Junglefowl. All data presented as mean and 95% confidence intervals with individual data points shown. For statistical analysis we used general linear modeling (GLM) considering species as a factor followed by Tukey posthoc test with a customary fiduciary significant level of $p<0.05$ (shown as dissimilar letters) in Minitab 17.

SUPPLEMENTARY INFORMATION

Supplementary Material and Methods

The data for Suppl.Figure 1 is from a compilation of heart and body mass data for adult birds through an extensive literature search in the Zoological Record (Thomson Reuters). Details on the compilation of the data can be obtained from our companion study (*1*). The full dataset of average heart mass and body mass per species, sex from the different scientific studies is available online (http://www.ifm.liu.se/biology/zoology/avian/staff/altimiras/birdheartdatabase/index.xml).

We obtained a pondered average heart mass per species by pooling data from males and females if available and pooling data from different studies. The pondered average was applied to give more weight to measurements obtained from multiple individuals than studies in which only one specimen was measured. We used the Jetz phylogeny to group all species in families (*2*). A family average was obtained for the bird families represented by nine or more species. Because of its relevance to the discussion we also grouped together three species in the superorder Ratites, the Ostrich, the Greater Rhea and the Emu.

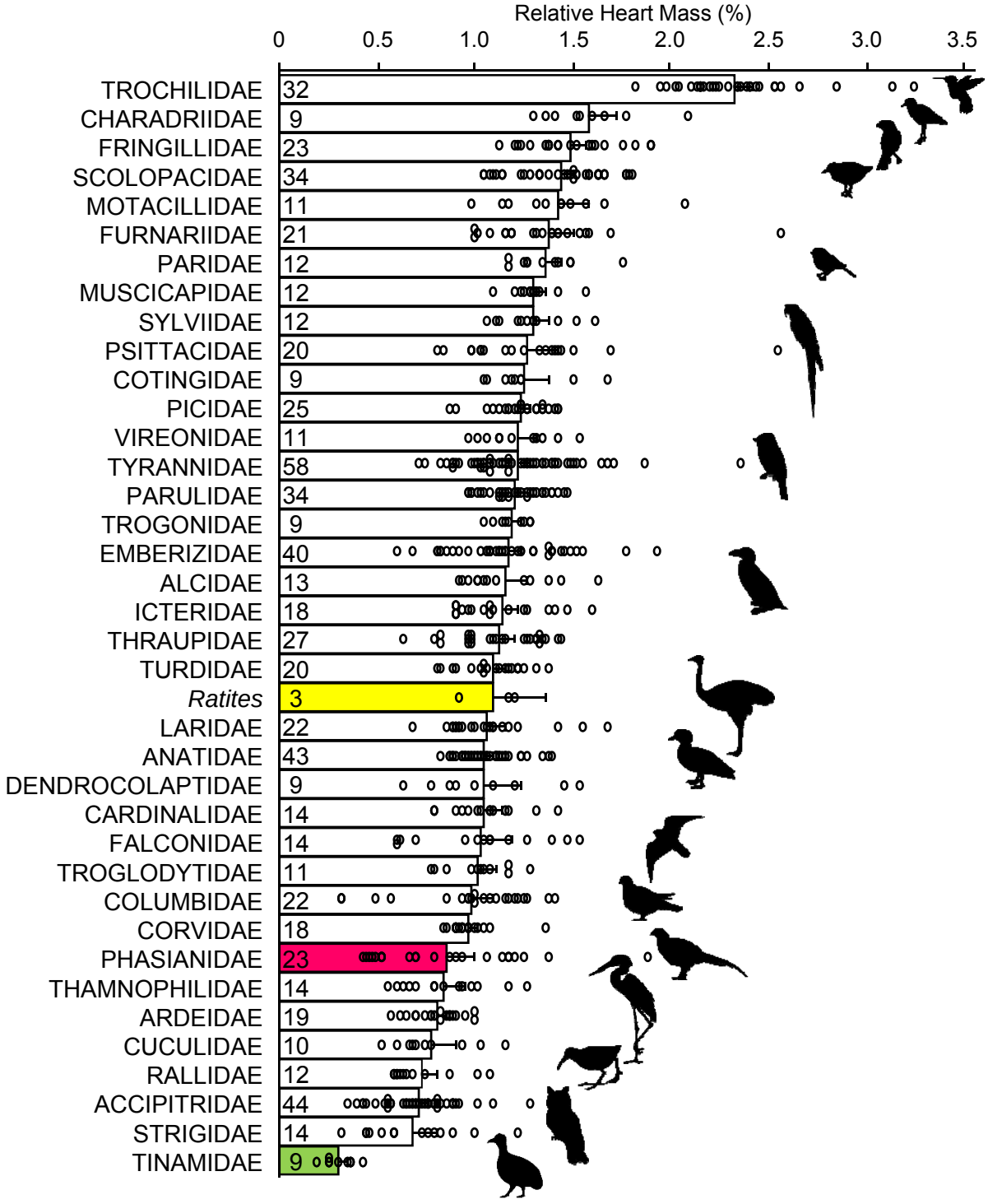
References

1. R. F. Nespolo, C. González-Lagos, J. J. Solano-Iguaran, M. Elfwing, A. Garitano-Zavala, S. Mañosa, J. C. Alonso, J. Altimiras, The adaptive evolution of flight mode and aerobic power in birds: a phylogenetic test of the heart-size hypothesis. *Physiol.Biochem.Zool.* **submitted**, (2017).
2. W. Jetz, G. H. Thomas, J. B. Joy, K. Hartmann, A. O. Mooers, The global diversity of birds in space and time. *Nature* **491**, 444-448 (2012).

Supplementary Table 1

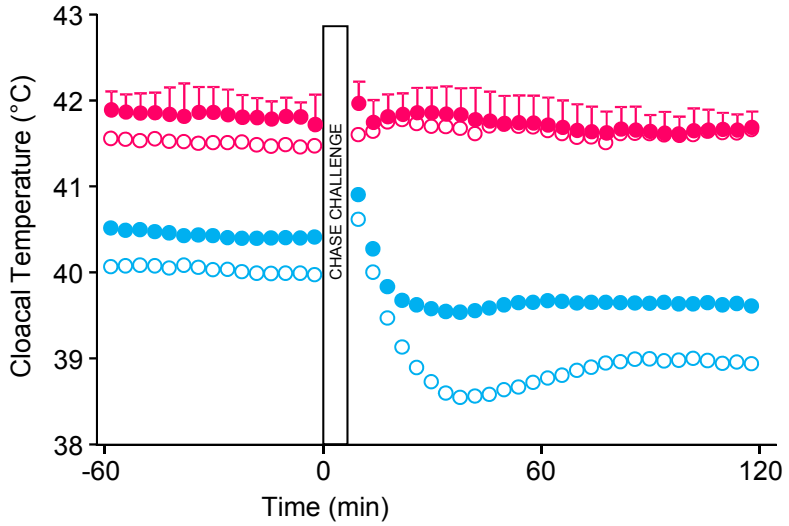
Gene identities, sequence IDs and primer sequences used in the analysis of gene expression displayed in Figure 7

Gene Name	**Description**	**Sequence ID**	**Forward Primer**	**Reverse Primer**
ERK2	*Gallus gallus* mitogen-activated protein kinase 1 (MAPK1), mRNA	NM_204150.1	CCAATGTGCTTCATCGCGACCT	CTGCAACACGAGCCAGTCCG
JNK1	*Gallus gallus* mitogen-activated protein kinase 8 (MAPK8), predicted mRNA	Transcripts X1-X6 XM_015288441.1 XM_004942133.2 XM_015288440.1 XM_015288439.1 XM_001233168.4 XM_004942132.2	GGCTGGGAACAGAATTTGGATG	ATTGTTGTCACGCTTGCTTCT
P38	*Gallus gallus* mitogen-activated protein kinase 14 (MAPK14), predicted mRNA	Transcripts X1-X2 XM_419263.4 XM_001232615.2	AGTGGGATGCATTATGGCTGA	GGGGTTCCAACGAGTCTCAA
PIK3CA	*Gallus gallus* phosphoinositide 3-kinase catalytic subunit, mRNA	NM_001004410.1	CTGCGGGGAAAGCGAGATGGA	CCATCCACCACAACAGAGCAGGC
TBP	*Gallus gallus* TATA-box binding protein , mRNA	NM_205103.1	GAACCACGTACTACTGCGCT	GCCAGTCTGGACTGTTCCTC
ACTB	*Gallus gallus* Actin, beta, mRNA	NM_205518.1	CACAGATCATGTTTGAGACCTT	CATCACAATACCAGTGGTACG
GAPDH	*Gallus gallus* Glyceraldehyde-3-phosphate dehydrogenase, mRNA	NM_204305.1	GTCAAGGCTGAGAACGGGAA	GCCCATTTGATGTTGCTGGG



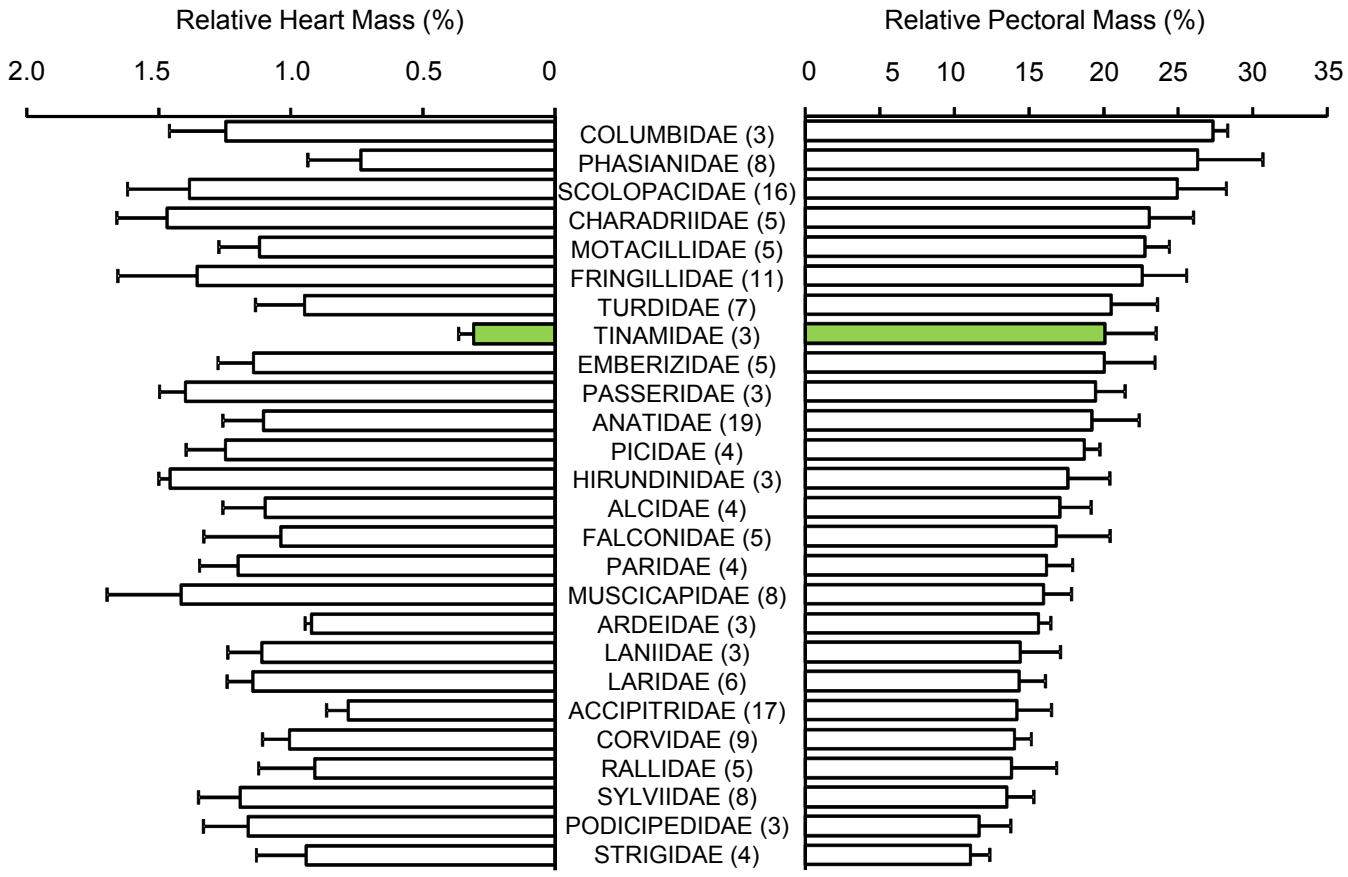
Suppl.Figure 1

Average relative heart mass in different bird families. Families shown are those represented by at least nine different species. Highlighted in color are the families Tinamidae (in green) and Phasianidae (in red, which includes the Red Junglefowl) and the superorder Ratites (in yellow), which is shown for its relevance to the discussion The number of species included in each family is shown at the base of the bar. Data shown as average and 95% confidence intervals, with the individual data points per species shown. Further details provided in Supplementary Material and Methods.



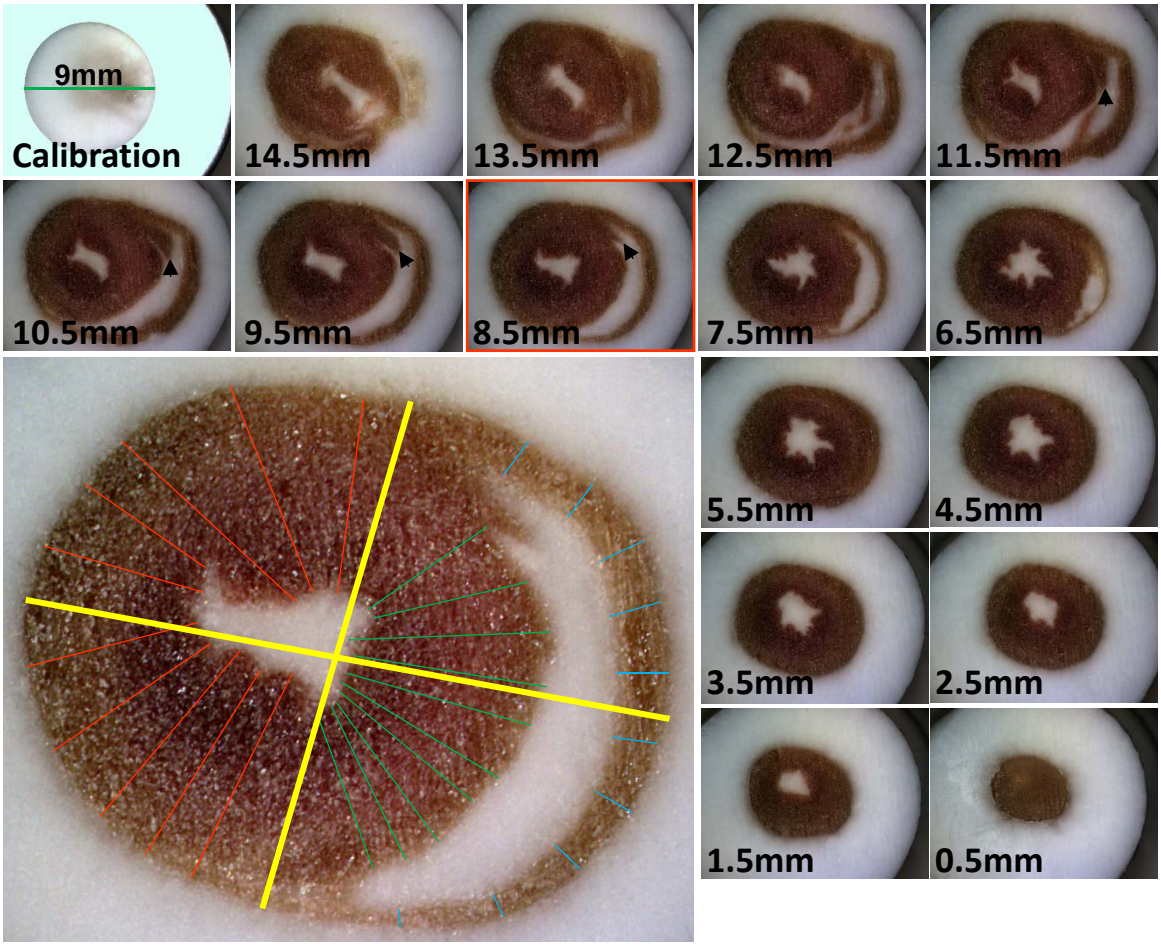
Supplementary Figure 2 (completes Figure 6 from the article)

Cloacal temperature before and after a 3 min chase-and-exhaust protocol in Ornate Tinamou (blue symbols) and domestic chickens (red symbols) kept at an ambient temperature of 4°C (open symbols) and 25°C (closed symbols). The chase-and-exhaust protocol was carried out after a baseline measurement lasting 1 h. Data from chickens at 25°C is presented fully and the data already shown in paper Figure 6 is displayed for comparison without standard deviations. No significant changes in cloacal temperature were observed in chickens at 25°C.



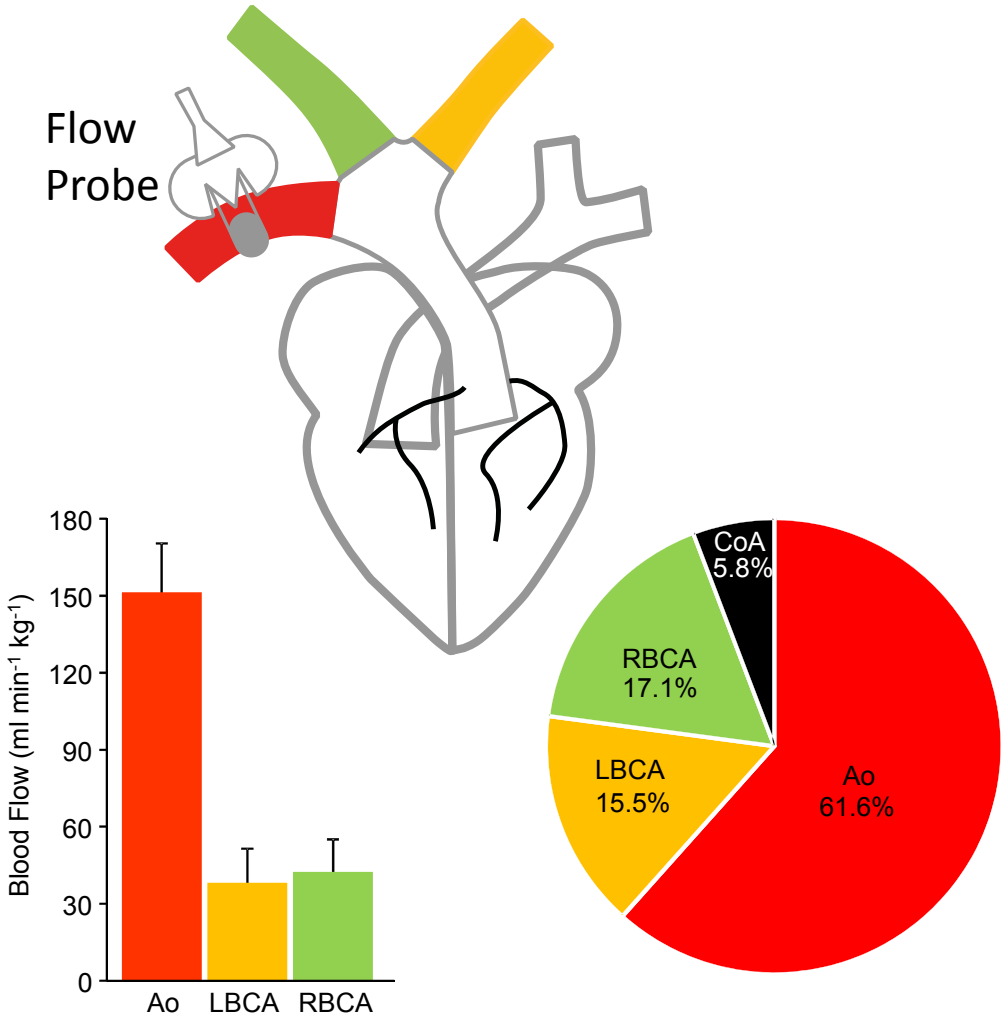
Suppl.Figure 3

Average relative heart mass (bars on the left) and relative pectoral mass (bars on the right) in different bird families represented by three or more species. Data obtained Magnan 1922 except data from Chilean and Ornate Tinamous, which are from our own measurements. Data shown as average and standard deviations. The number of species included in each family is shown in parenthesis.



Suppl.Figure 4

Pictorial description of the method for measuring ventricular wall thickness in the heart of a Chilean Tinamou *Nothoprocta perdicaria*. Sectioning was done in 500 um sections but only every other section is presented to show the entire heart together with one calibration picture. The sections used for analysis were two consecutive sections (only one shown, 8.5 mm from the apex of the heart) in which the right atrioventricular valve (RAVV) was still visible. Tip of the RAVV is shown by arrowheads. Notice that the RAVV becomes a muscular band with attachments on the ventral and dorsal side of the heart when closer to the base (seen in the sections 11.5 and 12.5 mm from the apex). The following measurements were taken: long and short axis (yellow lines) and 10 equidistributed measurement of the ventricular walls: free left ventricular wall (red lines), septal wall (green lines) and free right ventricular wall (blue lines). Free left ventricular wall and septal wall were averaged to represent the thickness of the left ventricle.



Supplementary Figure 5

Blood flow measurements in domestic chickens (N=4) carried out to estimate the contribution of brachiocephalic flow to total cardiac output. Flow in the left and right brachiocephalic arteries (LBCA and RBCA respectively) were measured in the same individuals after measuring flow in the aorta (Ao). Values shown as means and standard deviations. Total flows shown on the left graph. Relative flows (% of cardiac output) shown on the chart on the right. Flow in the coronary artery (CoA) was estimated as 5.8% of the total flow based on literature values in chickens. This value is likely to overestimate coronary flows in tinamous with smaller hearts, which will make the cardiac output measurements more conservative. Based on these measurements, the aortic flow values from the main study were corrected to account for brachiocephalic and coronary flows.