Received: 20 April 2022 | Revised: 17 May 2022 | Accepted: 1 June 2022

DOI: 10.1111/apha.13849

ACTA PHYSIOLOGICA

CARDIOVASCULAR PHYSIOLOGY

Regulation of heart rate following genetic deletion of the ß1 adrenergic receptor in larval zebrafish

William Joyce[1,2] | **Yihang K. Pan**[1] | **Kayla Garvey**[1] | **Vishal Saxena**[1] | **Steve F. Perry**[1]

[1]Department of Biology, University of Ottawa, Ottawa, Ontario, Canada

[2]Department of Biology – Zoophysiology, Aarhus Universitet, Aarhus C, Denmark

Correspondence
William Joyce, Department of Biology – Zoophysiology, Aarhus Universitet, C.F. Møllers Allé 3, 8000 Aarhus C, Denmark.
Email: william.joyce@bio.au.dk

Funding information
Natural Sciences and Engineering Research Council of Canada, Grant/Award Number: RGPIN 2017-05545; Novo Nordisk Fonden, Grant/Award Number: NNF19OC0055842

Abstract

Aim: Although zebrafish are gaining popularity as biomedical models of cardiovascular disease, our understanding of their cardiac control mechanisms is fragmentary. Our goal was to clarify the controversial role of the ß1-adrenergic receptor (AR) in the regulation of heart rate in zebrafish.

Methods: CRISPR-Cas9 was used to delete the *adrb1* gene in zebrafish allowing us to generate a stable *adrb1*$^{-/-}$ line. Larval heart rates were measured during pharmacological protocols and with exposure to hypercapnia. Expression of the five zebrafish *adrb* genes were measured in larval zebrafish hearts using qPCR.

Results: Compared with genetically matched wild-types (*adrb1*$^{+/+}$), *adrb1*$^{-/-}$ larvae exhibited ~20 beats min^{-1} lower heart rate, measured from 2 to 21 days post-fertilization (dpf). Nevertheless, *adrb1*$^{-/-}$ larvae exhibited preserved positive chronotropic responses to pharmacological treatment with AR agonists (adrenaline, noradrenaline, isoproterenol), which were blocked by propranolol (general ß-AR antagonist). Regardless of genotype, larvae exhibited similar increases in heart rate in response to hypercapnia (1% CO_2) at 5 dpf, but tachycardia was blunted in *adrb1*$^{-/-}$ larvae at 6 dpf. *adrb1* gene expression was abolished in the hearts of *adrb1*$^{-/-}$ larvae, confirming successful knockout. While gene expression of *adrb2a* and *adrb3a* was unchanged, *adrb2b* and *adrb3b* mRNA levels increased in *adrb1*$^{-/-}$ larval hearts.

Conclusion: Despite *adrb1* contributing to the setting of resting heart rate in larvae, it is not strictly essential for zebrafish, as we generated a viable and breeding *adrb1*$^{-/-}$ line. The chronotropic effects of adrenergic stimulation persist in *adrb1*$^{-/-}$ zebrafish, likely due to the upregulation of other ß-AR subtypes.

KEYWORDS
adrenaline, cardiac, catecholamine, GPCR, hypercapnia

1 | INTRODUCTION

The genomes of most vertebrates contain three ß-adrenergic receptors (ß-ARs), ß1-AR, ß2-AR, and ß3-AR, encoded by the *adrb1*, *adrb2*, and *adrb3* genes, respectively. These paralogs originated from a single ancestral gene following two rounds of whole-genome duplication early in the evolution of vertebrates.[1,2] Different ß-AR

isoforms display characteristic pharmacological properties[3–5] and exhibit tissue-specific expression patterns, although co-expression of all three receptors occurs in some organs. In the healthy mammalian heart, ß1-ARs are predominantly expressed, but it is complemented by expression of ß2-ARs and ß3-ARs at least in a subpopulation of cardiomyocytes.[6–9] During heart failure, ß1-ARs are downregulated and ß2-ARs become relatively more abundant and of greater functional relevance.[10,11] The relative expression of ß2/ß1 receptors is higher in the cardiomyocytes of the sino-atrial node (SAN), which harbors the dominant cardiac pacemaker, than surrounding atrial or ventricular myocardium.[12–14]

Zebrafish are emerging as popular model organisms for understanding heart function, including the effects of ß-adrenergic stimulation.[15–20] Owing to a third whole-genome duplication event in early teleost fishes, zebrafish are endowed with a bolstered array of *adrb* genes: *adrb1*, *adrb2a*, *adrb2b*, *adrb3a*, and *adrb3b*.[2,21] Evidently, the "second" *adrb1* gene was lost, presumably as it was redundant; such "nonfunctionalization" is a common fate of duplicated genes.[22] Previously, pharmacological and morpholino-based knockdown approaches[15,21] were used to characterize the roles of the different ß-AR isoforms in zebrafish, including their importance in setting heart rate. In general, the results of these studies indicate a leading role for the ß1-AR in determining the normal positive chronotropic response to adrenergic stimulation. The preferential reliance on the ß1-AR is consistent with it being the most highly expressed *adrb* gene in the adult zebrafish heart.[15,21] In larval [3 and 4 days post-fertilization (dpf)] zebrafish, morpholino knockdown of *adrb1* reduced heart rate and blunted the positive chronotropic responses induced by adrenaline or isoproterenol (ß-AR agonists),[15,21] while *adrb2a* and *adrb2b* knockdown increased baseline heart rate.[15] In 5 dpf larvae, *adrb1* knockdown and ß1-AR-specific antagonist atenolol abolished the tachycardia in response to elevated CO_2 (hypercapnia) exposure[23] while atenolol reduced heart rate and cardiac contractility in adult zebrafish hearts.[24] These data are somewhat surprising as previous studies suggested that ß2-ARs are dominantly expressed in atrium and ventricle in other teleost fish species.[25–30] Moreover, an immunohistological investigation showed abundant ß2-AR protein expression across the zebrafish heart, including the SAN region.[16]

Pharmacological and morpholino knockdown approaches can be limited by non-specific and off-target effects.[31] The generation of *adrb* knockout mice has previously proven instrumental in delineating the roles of specific receptors in mammals.[32] Mice lacking the ß1-AR (*adrb1*$^{-/-}$) exhibit high embryonic lethality (~70%–90% mortality), but those that do survive to adulthood appear phenotypically normal.[33] Despite evidence for enriched ß2-AR expression in the sinoatrial node cardiomyocytes of mammals,[14] which has functional significance on the ion currents that determine the rate of spontaneous depolarization,[12,13] *adrb1*$^{-/-}$ mice lack a chronotropic, as well as inotropic, response to ß-AR stimulation with isoproterenol.[33]

The goal of the present investigation was to generate an *adrb1*$^{-/-}$ zebrafish line to clarify the importance of the ß1-AR in adrenergic control of heart rate in this popular model species. CRISPR-Cas9 gene editing was used to delete the single *adrb1* exon, after which a suite of protocols was conducted to study heart rate regulation in *adrb1*$^{-/-}$ larval zebrafish.

2 | RESULTS

2.1 | The generation of a viable *adrb1*$^{-/-}$ line

CRISPR-Cas9 was used to delete the single exon of *adrb1* in zebrafish (Figures 1 and S1). To establish whether wild-type and knockout larvae exhibited equivalent rates of survival, four separate F1 *adrb1*$^{+/-}$ incrosses (1 male 1 female) were performed and the genotypes of the offspring were determined at 7 and 28 dpf. These results were compared with the expected percentage of each genotype based on Mendelian ratios (*adrb1*$^{+/+}$ 25%, *adrb1*$^{+/-}$ 50%, *adrb1*$^{-/-}$ 25%). At both developmental time points, there were no significant differences from expected Mendelian ratios (Figure S2; $p = 0.06$ at 7 dpf; $p = 0.12$ at 28 dpf). Indeed, while at 7 dpf, the *adrb1*$^{-/-}$ genotype tended to be underrepresented (13.8%), at 28 dpf it was correspondingly overrepresented (41.7%), apparently due to *adrb1*$^{+/+}$ and *adrb1*$^{+/-}$ being removed from the population by chance at 7 dpf (Figure S2). As such, when the 7 and 28 dpf were pooled to provide an overview of the general population across larval development (Figure 1C), presuming that the individuals sampled at 7 dpf would have survived in similar proportions to those left until 28 dpf, the distribution of genotypes was very close to theoretical Mendelian ratios ($p = 0.43$; *adrb1*$^{+/+}$ 24.0%, *adrb1*$^{+/-}$ 55.8%, *adrb1*$^{-/-}$ 20.2%). Anecdotally, in the propagated F2 lines, there was no excess mortality in *adrb1*$^{-/-}$ fish, which reached reproductive age (~90 dpf) at the same time as *adrb1*$^{+/+}$ siblings.

2.2 | Heart rate during development

Throughout development, there was an overall effect of genotype ($F(1, 453) = 136.0$; $p < 0.001$), wherein *adrb1*$^{-/-}$ larvae generally exhibited heart rates ~20 beats min^{-1} lower than *adrb1*$^{+/+}$ larvae (Figure 2). There was an overall effect of age ($F(8, 453) = 112.9$; $p < 0.001$) but no genotype*age interaction ($F(8, 453) = 1.21$; $p = 0.29$) as

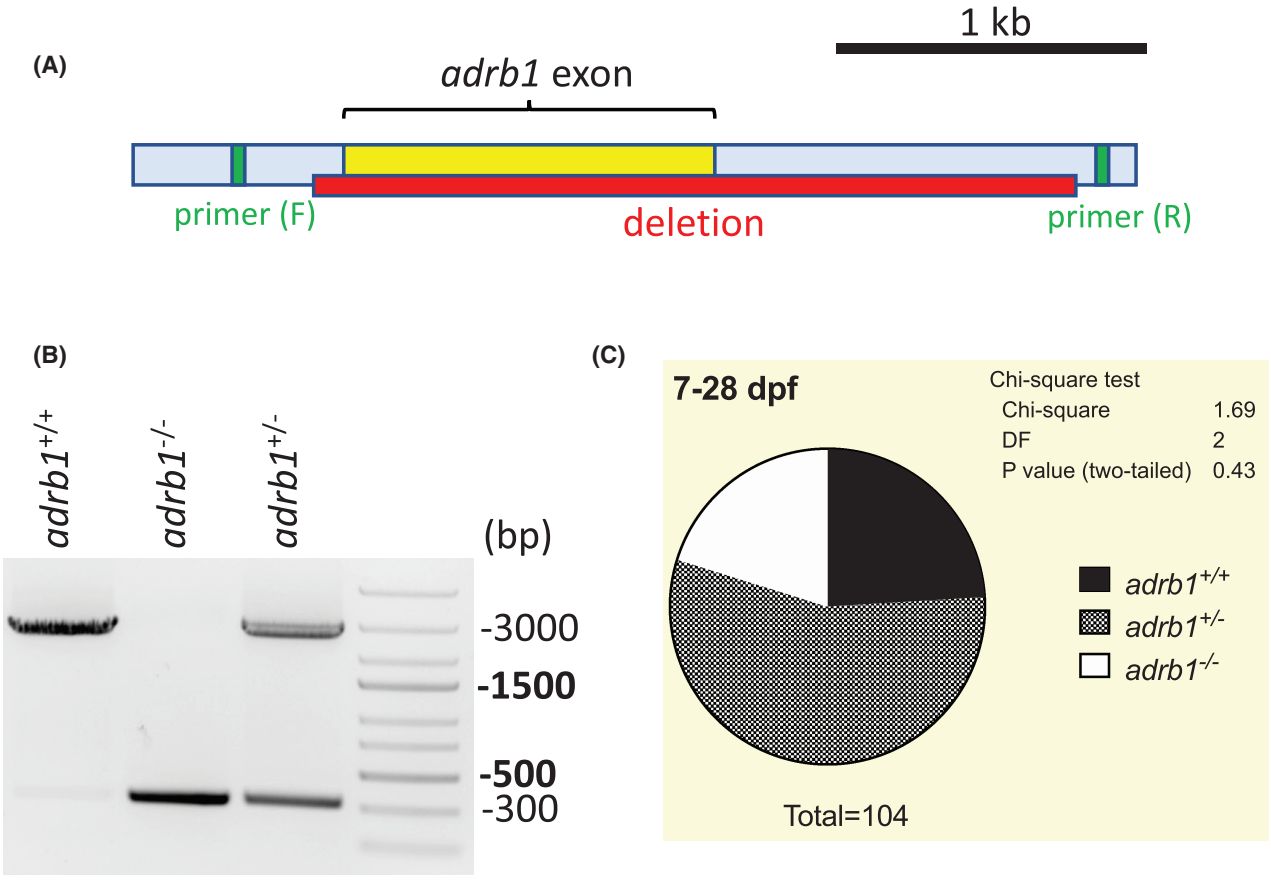

FIGURE 1 The generation and screening of an *adrb1* knockout ($adrb1^{-/-}$) zebrafish line. (A) Schematic illustration of the *adrb1* gene within chromosome 12 including the single exon, the deleted segment (confirmed by Sanger sequencing), and sequencing primer targets. See Supplementary Material for annotated sequence. (B) Polymerase chain reaction with the sequencing primers generated a ~3000 bp wild-type band ($adrb1^{+/+}$), a ~300 bp mutant band only ($adrb1^{-/-}$) or both bands ($adrb1^{+/-}$). (C) The results of F1 heterozygote ($adrb1^{+/}$) incrosses generated offspring in the expected Mendelian ratio (compared with Chi-square test, $p = 0.43$; $adrb1^{+/+}$ 24.04%, $adrb1^{+/-}$ 55.77%, $adrb1^{-/-}$ 20.19%). Note that this figure includes pooled data from the populations sampled at 7 and 28 dpf; for each subsample (age) see Figure S2.

both genotypes exhibited parallel triphasic changes consisting of an increase in heart rate from 2 to 5 dpf, a decline from 6 to 11 dpf, and a subsequent increase from 11 to 21 dpf (Figure 2).

2.3 | Heart rate responses to adrenergic agonists and antagonists

Adrenaline (Figure 3A; $F(1, 84) = 65.43$; $p < 0.001$), noradrenaline (Figure 3B; $F(1, 84) = 14.93$; $p < 0.001$), and isoproterenol (Figure 3C; $F(1, 84) = 66.46$, $p < 0.001$) each caused an increase in heart rate. While in each analysis, there was a significant (adrenaline; $p = 0.02$) or near significant (noradrenaline and isoproterenol respectively; $p = 0.06$, $p = 0.07$) overall effect of genotype, in no case was there a genotype*agonist interaction, which indicates that $adrb1^{-/-}$ larvae responded to the adrenergic agonists in a similar manner as $adrb1^{+/+}$ larvae. Thus, the extent of tachycardia in response to adrenergic agonists was independent of genotype. For example, in $adrb1^{+/+}$ larvae, untreated larvae had heart rates of 196.0 ± 9.5 beats min^{-1} (mean ± SEM), which rose to 240 ± 9.0 beats min^{-1} after isoproterenol (44 beats min^{-1} increase). By comparison, untreated $adrb1^{-/-}$ larvae had heart rates of 169.5 ± 7.3 beats min^{-1}, which rose to 219.5 ± 9.6 beats min^{-1} after isoproterenol (50 beats min^{-1} increase).

The adrenergic antagonists had strong and statistically significant overall effects ($p < 0.001$, providing a source of ~50% of variation in the models), however only propranolol, and not sotalol, appeared to attenuate the responses to the agonists (Figure 3). The mechanistic basis for the superior ß-blocking action of propranolol than sotalol remains unclear, and should be addressed in future pharmacological investigations. In each analysis, there was a genotype*antagonist interaction ($p < 0.05$), as propranolol abolished the differences in heart rate exhibited between the genotypes. For instance, after propranolol treatment, heart rate in $adrb1^{+/+}$ larvae was 114.0 ± 13.1 beats min^{-1} and in $adrb1^{-/-}$ larvae was 125.5 ± 5.8 beats min^{-1}.

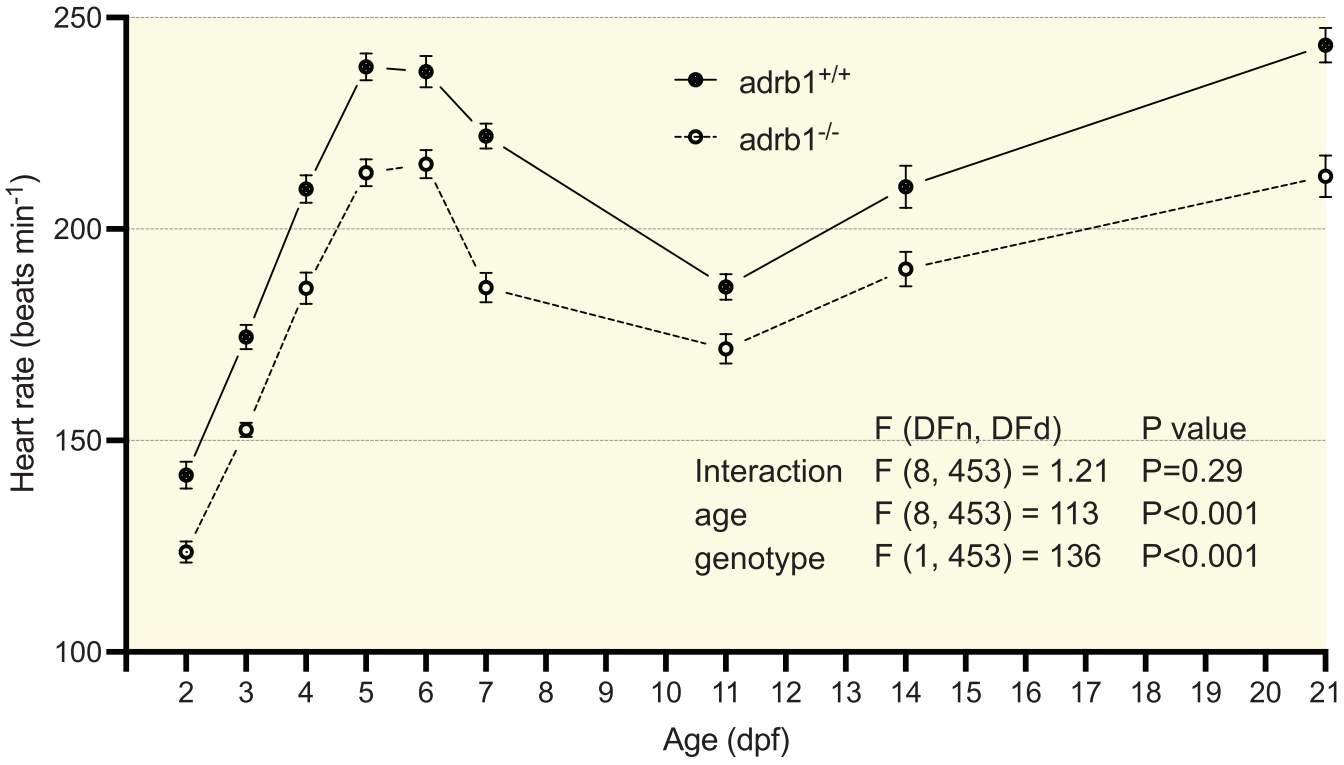

FIGURE 2 Heart rate during development in unanesthetized *adrb1*$^{+/+}$ and *adrb1*$^{-/-}$ larvae. Heart rates were optically measured from 2–21 days post-fertilization (dpf). Each individual larva was used at only one time point. Statistical results show the output of a two-way ANOVA. *N* values were 16–55 at each time point. Data points show means ± SEM.

DMSO (vehicle) exerted a small (accountable for 6.7% variation in the model), but statistically significant overall negative chronotropic effect on heart rate (Figure 4A; $F(1, 51) = 6.60$; $p = 0.01$). Atenolol (ß1 receptor antagonist) had no overall effect ($F(1, 46) = 2.26$, $p = 0.14$). The ß2 receptor antagonist ICI-118551 ($F(1, 54) = 79.47$, $p < 0.001$) and the ß3 receptor antagonist SR59230A ($F(1, 54) = 63.12$, $p < 0.001$) exerted much more pronounced negative chronotropic effects (each accounting for ~44% variation in model). Isoproterenol again exerted strong and statistically significant ($p < 0.001$) positive chronotropic effects in both genotypes, yet there were no significant ($p > 0.05$) genotype*agonist or genotype*antagonist interactions in any of the models. None of the ß-AR specific antagonists reduced the effect of isoproterenol (agonist*antagonist interactions, $p > 0.05$). Despite the pronounced lowering of heart rate caused by the ß3 receptor antagonist SR59230A, addition of the ß3 receptor agonist BRL-37344 was without effect (Figure S3: $F(1, 22) = 0.90$; $p = 0.35$) in either genotype (interaction, $F(1, 22) = 0.19$, $p = 0.67$).

2.4 | Heart rate responses to hypercapnia

In 5 dpf larvae, there was a strong effect of hypercapnia over time ($F(8, 128) = 104.0$, $p < 0.001$), but no overall effect of genotype ($F(1, 16) = 1.68$, $p = 0.21$) or genotype*time interaction ($F(8, 128) = 0.13$, $p > 0.99$) (Figure 5A). The maximum effect manifested in the first measurement period after hypercapnia exposure commenced (Figure 5A), resulting in a 75.7 ± 7.7 beats min^{-1} acceleration of heart rate in *adrb1*$^{+/+}$ and a 79.2 ± 12.6 beats min^{-1} acceleration of heart rate in *adrb1*$^{-/-}$ larvae (Figure 5B). At 6 dpf, there was also a significant effect of hypercapnia exposure over time ($F(8, 152) = 87.30$, $p < 0.001$) and additionally an overall effect of genotype ($F(1, 19) = 7.884$, $p = 0.01$), whereby starting heart rates were ~20 beats min^{-1} lower in *adrb1*$^{-/-}$ than *adrb1*$^{+/+}$ larvae. Notably, there was also a genotype*time interaction ($F(8, 152) = 4.162$, $p < 0.001$) which could be ascribed to the fact that *adrb1*$^{+/+}$ larvae exhibited a greater peak increase ($p < 0.05$) in heart rate at the onset of hypercapnia than *adrb1*$^{-/-}$ (78.3 ± 8.0 beats min^{-1} vs. 51.0 ± 7.2 beats min^{-1} increase) (Figure 5D).

2.5 | The effects of isoproterenol on isolated hearts in vitro

Our series of in vivo procedures indicated that the hearts of *adrb1*$^{-/-}$ larvae retained the capacity to respond to adrenergic stimulation with an increase in heart rate. However, this approach could not distinguish whether

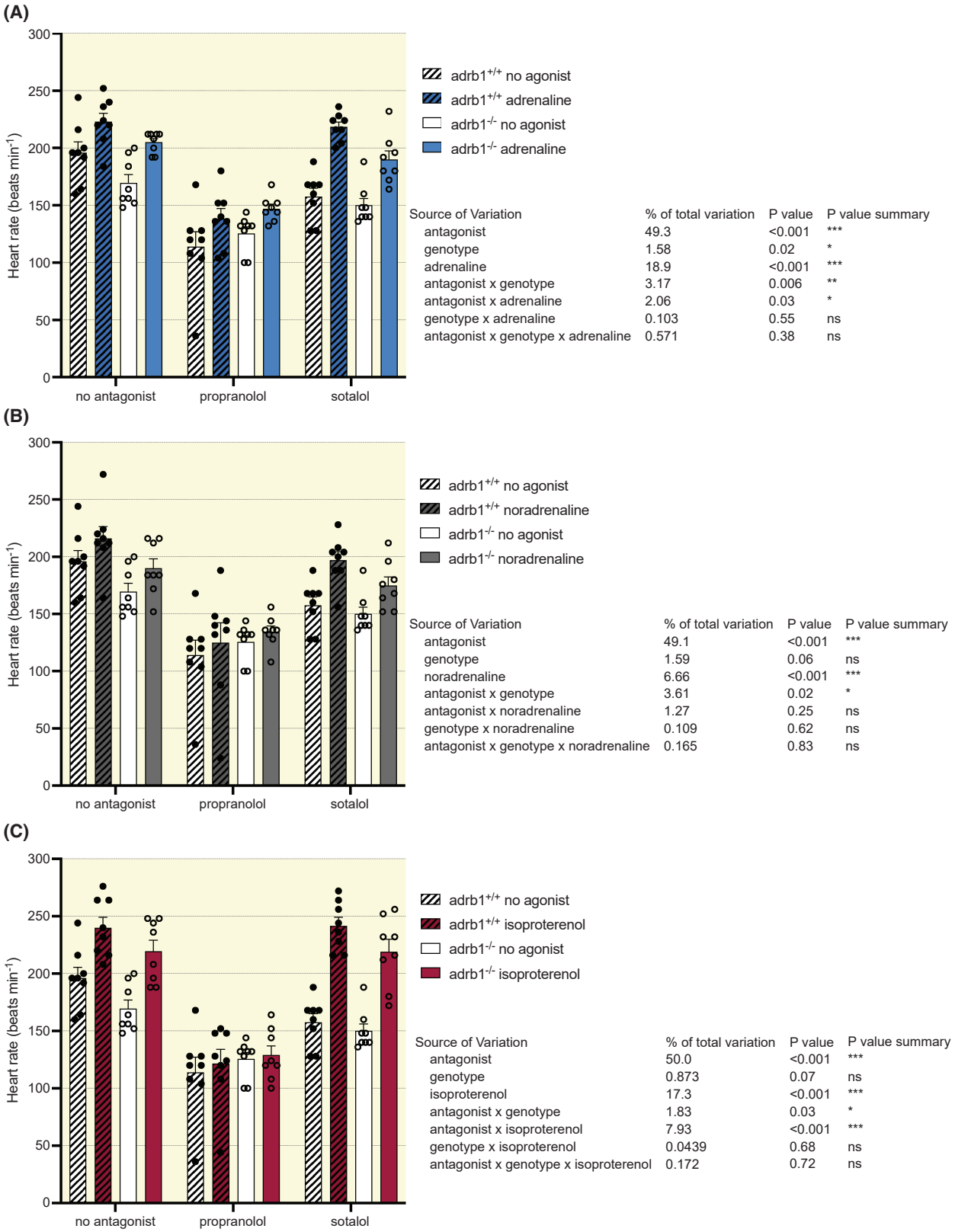

FIGURE 3 The effect of different adrenergic agonists (A, adrenaline; B, noradrenaline; C, isoproterenol) and general ß-AR antagonists (propranolol or sotalol) on heart rate in 7 dpf *adrb1*$^{+/+}$ and *adrb1*$^{-/-}$ zebrafish larvae. All drugs were applied at a final concentration of 100 µM. The effects of each agonist were analyzed in separate three-way ANOVAs. Note that on each panel the "no agonist" bars (non-colored), including those with antagonists, are repeated to provide control data for comparison with a given agonist; all of the data were collected in the same sessions so the use of the same controls was appropriate. ns, $p > 0.05$; *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$. *N* values are shown by individual data points. Bars show means ± SEM.

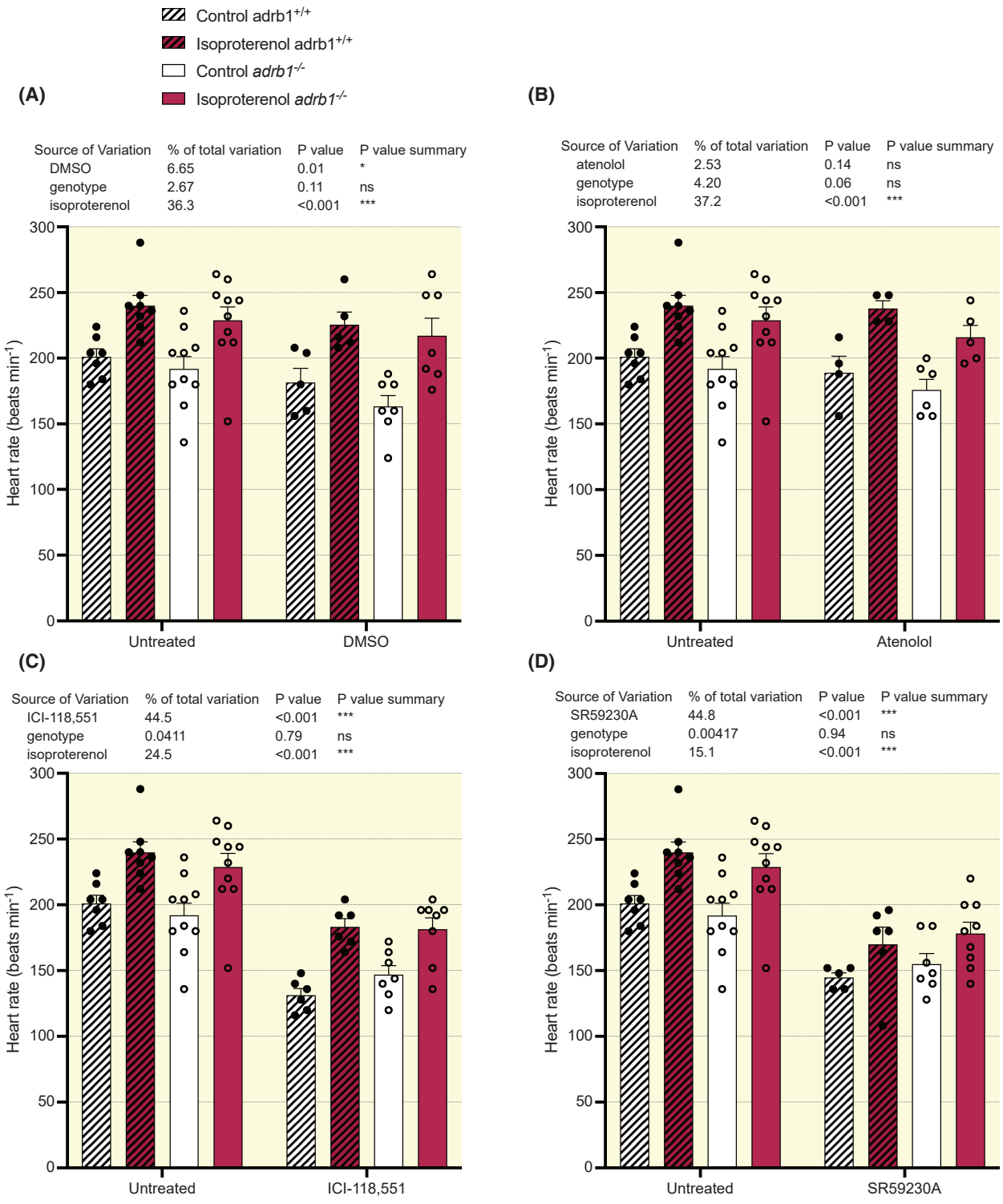

FIGURE 4 The effects of DMSO vehicle (A) and different adrenergic antagonists (B, atenolol, ß1-specific; C, ICI-118551, ß2-specific; D, SR59230A, ß3-specific) and the ß-AR agonist (isoproterenol) on heart rate in 7 dpf *adrb1*$^{+/+}$ and *adrb1*$^{-/-}$ zebrafish larvae. All drugs, except SR59230A (10 μM) were applied at a final concentration of 100 μM. The effects of each antagonist were analyzed in separate three-way ANOVAs. Note that on each panel the four "untreated" bars (i.e., no antagonist) are repeated to provide control data for comparison with a given antagonist; all of the data were collected in the same sessions so the use of the same controls was appropriate. ns, $p > 0.05$; *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$. *N* values are shown by individual data points. Bars show means ± SEM.

the tachycardia arose from a direct effect on the heart, as opposed to affecting vascular tone, which could affect heart rate indirectly through possible changes in cardiac filling.[34] To resolve this, we studied the effects of isoproterenol on heart rate in the spontaneously-beating isolated heart in vitro. Because it was not possible to reliably

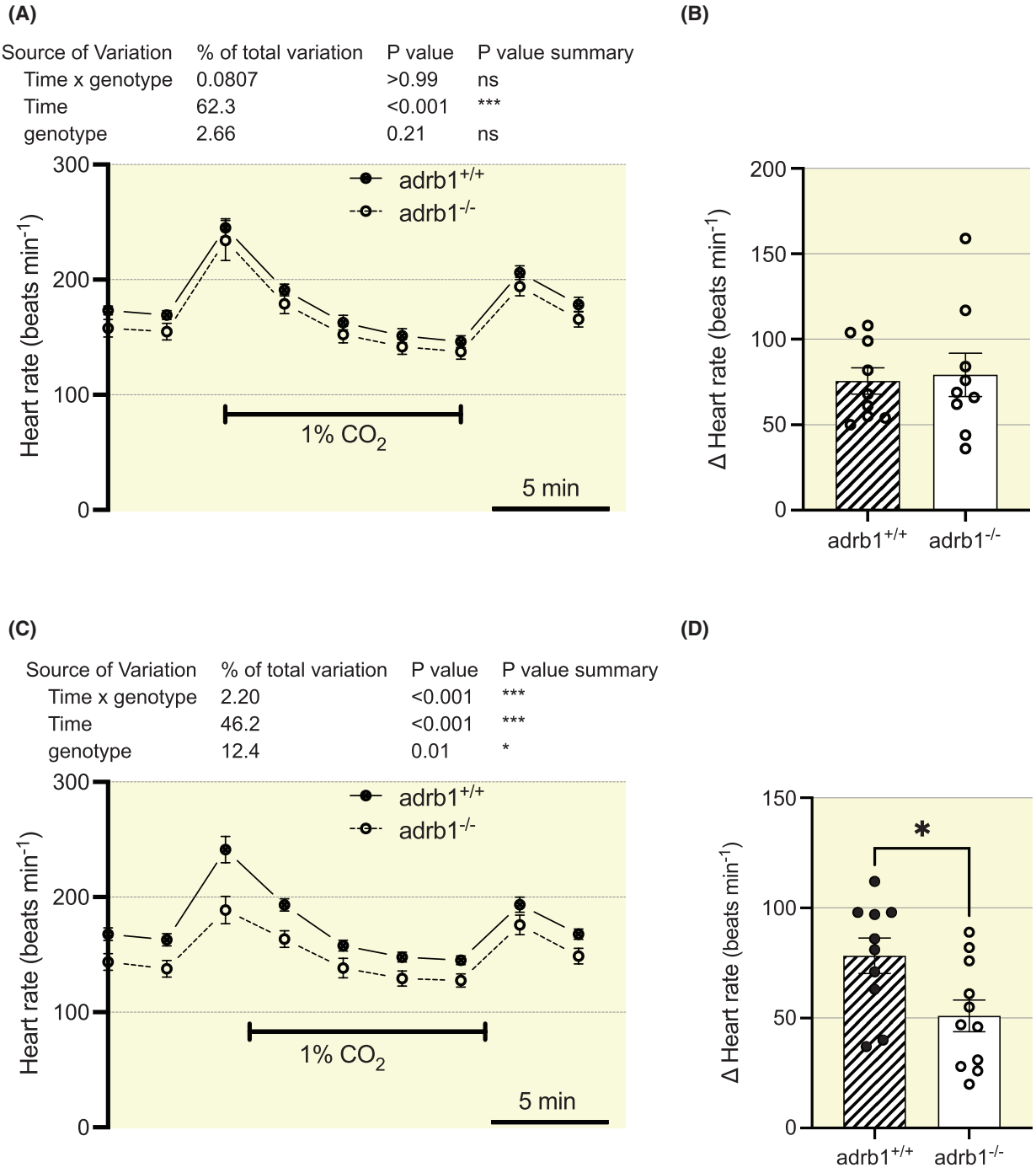

FIGURE 5 The effect of sustained hypercapnia (10 min) on heart rate in 5 (A, B) and 6 (C, D) dpf *adrb1*$^{+/+}$ and *adrb1*$^{-/-}$ zebrafish larvae. Statistical results are the output of a repeated-measures two-way ANOVA (A, C). In panels B and D, the maximum changes in heart rate were calculated for each individual immediately after the onset of hypercapnia; genotypes were compared using unpaired *t*-test. ns, $p > 0.05$; *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$. $N = 9$–11. Data points show means ± SEM (A, C), bars show means ± SEM and individual points (B, D).

perform these experiments on small larval hearts, we instead used hearts from adult (3 month post-fertilization) *adrb1*$^{+/+}$ and *adrb1*$^{-/-}$ zebrafish. There was no significant difference ($t = 0.51$, df = 10, $p = 0.62$) in body mass of adult fish between the genotypes (*adrb1*$^{+/+}$, 0.20 ± 0.03 g; *adrb1*$^{-/-}$, 0.24 ± 0.07 g). There was an overall significant positive chronotropic effect of isoproterenol treatment (F (1, 10) = 17.1, $p = 0.002$) but no effect of genotype (F (1, 10) = 0.01, $p = 0.92$) or isoproterenol*genotype interaction (F (1, 10) = 0.37, $p = 0.56$), showing that the isolated *adrb1*$^{-/-}$ heart responded to adrenergic stimulation in an identical manner to *adrb1*$^{+/+}$ (Figure 6).

2.6 | Cardiac gene expression in larval zebrafish

To understand how *adrb1*$^{-/-}$ larvae were capable of preserved responses to adrenergic agonists and hypercapnia (which was blunted but not abolished at 6 dpf), we measured the expression of each of the five zebrafish adrb genes (*adrb1*, *adrb2a*, *adrb2b*, *adrb3a*, and *adrb3b*) in the hearts of 7 dpf *adrb1*$^{+/+}$ *and adrb1*$^{-/-}$ larvae (Figure 7).

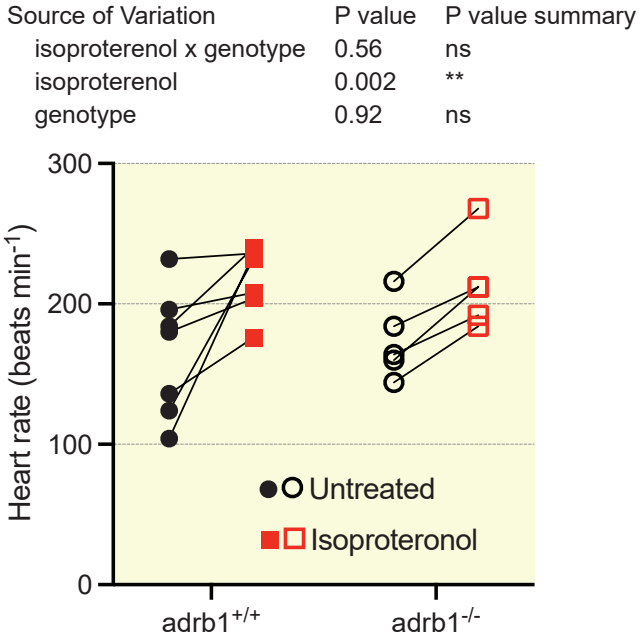

FIGURE 6 The effect of isoproterenol (final concentration 1 μM) on heart rate in spontaneously-beating isolated hearts from *adrb1*$^{+/+}$ and *adrb1*$^{-/-}$ adult (3 months post-fertilization) zebrafish. Statistical results are the output of a repeated-measures two-way ANOVA. ns, $p > 0.05$; *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$. $N = 5$–7. Data are presented as individual data points.

This experiment verified that, as a result of the genomic deletion, *adrb1* expression was abolished in the hearts of *adrb1*$^{-/-}$ larvae. While expression of *adrb2a* and *adrb3a* was unchanged in the *adrb1* knockouts, both *adrb2b* ($t = 2.63$, df = 10, $p = 0.03$) and *adrb3b* ($t = 3.04$, df = 10, $p = 0.01$) expressions were increased in *adrb1*$^{-/-}$ relative to *adrb1*$^{+/+}$ larvae, demonstrating differential regulation of gene transcription, potentially as a form of genetic compensation, for loss of the ß1-AR.

2.7 | Respirometry

To ascertain whether the reduced routine heart rates of *adrb1*$^{-/-}$ larvae (Figure 2) were associated with altered oxygen consumption during normoxia or hypoxia (measured as critical oxygen partial pressure; P_{crit}), microrespirometry was conducted on 7 dpf *adrb1*$^{+/+}$ and *adrb1*$^{-/-}$ larvae. Neither routine oxygen consumption ($t = 0.18$, df = 42, $p = 0.86$) nor P_{crit} ($t = 1.06$, df = 34, $p = 0.30$) was different between genotypes (Figure 8).

3 | DISCUSSION

Although the results of some previous studies[15,23,24] suggested that the ß1-AR (encoded by *adrb1*) is largely responsible for positive chronotropic effects of adrenergic stimulation in zebrafish, as is believed to be the case in mammals,[33] this notion is in contrast to the long held dogma that ß2-ARs dominate in the fish heart.[28] Our results indicate that, while deletion of *adrb1* reduces routine heart rate, it is not essential for positive chronotropic responses to pharmacological adrenergic stimulation or environmental stressors that increase adrenergic tone. The preserved adrenergic tachycardia may be facilitated

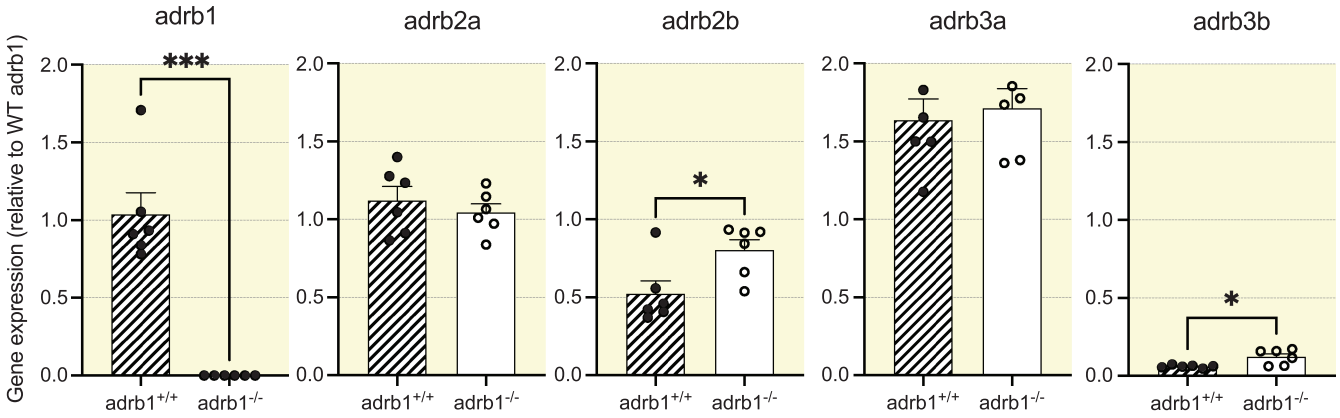

FIGURE 7 Expression of five *adrb* genes in 7 dpf *adrb1*$^{+/+}$ and *adrb1*$^{-/-}$ zebrafish hearts analyzed with qPCR. Statistical comparisons made with unpaired *t*-test. *$p < 0.05$; ***$p < 0.001$. For each sample $N = 6$ pools of 20 hearts. Bars show means ± SEM and individual data points.

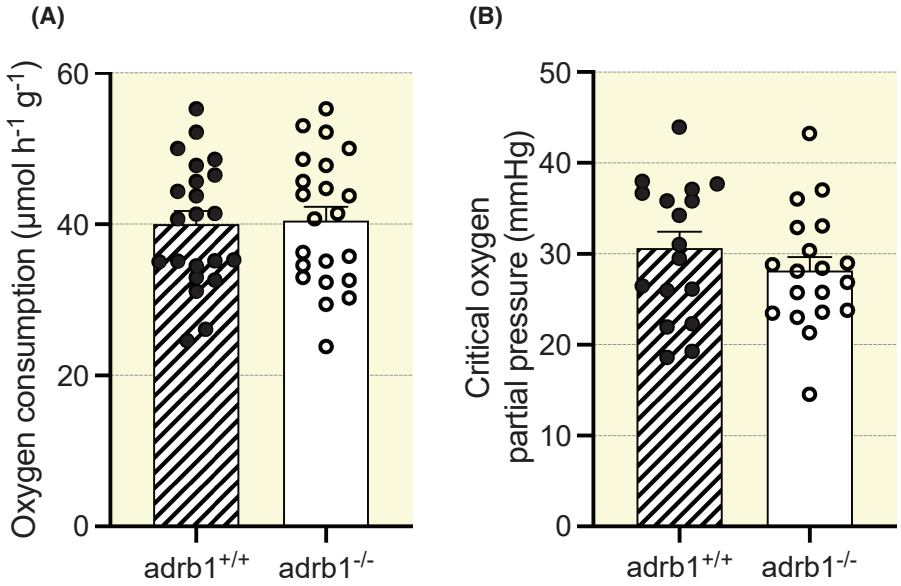

FIGURE 8 Routine oxygen consumption (A) and critical oxygen partial pressure (P_{crit}; B) in *adrb1*$^{+/+}$ and *adrb1*$^{-/-}$ zebrafish determined with respirometry. Oxygen consumption measurements $N = 22$, successful P_{crit} measurements $N = 17–19$. There were no significant differences in either parameter between genotypes. Bars show means ± SEM and individual data points.

by upregulation of other ß-ARs, namely those encoded by *adrb2b* and/or *adrb3b*.

Unlike in mice, in which deletion of the *adrb1* results in high embryonic lethality,[33] we observed that *adrb1*$^{-/-}$ zebrafish survived in expected Mendelian ratios and appeared outwardly as phenotypically normal and viable adults. Body mass of 7 dpf larvae (see Respirometry methods) and 3 month post-fertilization adults (see The effects of isoproterenol on isolated hearts in vitro Results) were identical. Despite exhibiting lower heart rates, 7 dpf *adrb1*$^{-/-}$ larvae exhibited equivalent rates of oxygen consumption to their *adrb1*$^{+/+}$ cousins. As internal convection is essential for normal aerobic metabolism, even in early larval zebrafish,[35] the maintained rate of oxygen consumption may be supported by increased stroke volume and/or elevated arterio-venous oxygen extraction, according to the Fick principle.[36,37]

In agreement with previous work in zebrafish,[38,39] heart rate acutely rose from 2 to 5 dpf, a pattern which has been attributed to increased circulating catecholamine levels exerting increasing ß-adrenergic tone.[40] Thus, a blunting of cardiac acceleration during early development was anticipated in *adrb1*$^{-/-}$ larvae, yet they exhibited a parallel rise in heart rate. Either the potentially increased adrenergic tone can stimulate other ß-ARs or the increase in heart rate may represent intrinsic remodeling on the sino-atrial pacemaker. There was a clear difference in heart rate between genotypes even at 2 dpf, which is consistent with studies showing that heart rate is increased with isoproterenol treatment at 2 or 3 dpf.[17,38,41] Importantly, the finding of the current study of reduced heart rate in the *adrb1*$^{-/-}$ larvae at 2 dpf indicates that cardiac ß-adrenergic tone may start earlier than traditionally believed (after 5 dpf) as suggested by onset of propranolol sensitivity.[39] This is consistent with the previous finding that some of the enzymes necessary for catecholamine synthesis (i.e. tyrosine hydroxylase and dopamine ß-hydroxylase) are expressed by 2 dpf, at which point endogenous whole-body noradrenaline also becomes measurable in larval zebrafish.[42]

Arguably, the most striking finding of the present study was that deletion of *adrb1* did not alter the chronotropic responses to ß-adrenergic receptor agonists, clearly indicating that other receptors can mediate chronotropic responses to catecholaminergic stimulation in the zebrafish heart. Because the pharmacological treatments may have induced supra-physiological levels of adrenergic stimulation, we subsequently studied the heart rate responses to an environmentally relevant stressor, hypercapnia, which is believed to increase adrenergic tone.[23] However, *adrb1*$^{-/-}$ larvae exhibited the typical increase in heart rate during hypercapnia, identical to *adrb1*$^{+/+}$ at 5 dpf albeit significantly blunted at 6 dpf (Figure 5D). The unaltered response to CO_2 at 5 dpf was unexpected given that in a previous study, the hypercapnic tachycardia at this developmental time point was abolished by morpholino knockdown of *adrb1* or pre-treatment with an ß1-AR-specific ß-blocker (atenolol).[23] Because the zebrafish heart can respond to increased stretch (i.e. hemodynamic load) with an increased beating rate,[34] and adrenergic stimulation may increase venous return independently of activating cardiac ß1-ARs,[43] we considered the possibility that the

preserved responses of the *adrb1*$^{-/-}$ line to adrenergic stimulation could be attributable to an indirect effect on the heart. However, we showed that the spontaneously beating isolated heart from adult *adrb1*$^{-/-}$ zebrafish responded to isoproterenol stimulation in an identical manner to *adrb1*$^{+/+}$, confirming that the knockout indeed displays a preserved intrinsic capacity to respond to adrenergic stimulation with an increase in heart rate (Figure 6). Because this experiment was conducted in adult hearts, it suggests the general mechanisms we have uncovered in larvae are likely translatable to later life stages. However, in the future, dedicated experiments will be required to understand fully cardiac control in adult *adrb1*$^{-/-}$ zebrafish.

We hypothesize that the sustained hypercapnic tachycardia is a consequence of genetic compensation, i.e. upregulation of related receptors to mitigate the effect of knockout.[31] For instance, it was previously shown that induction of a deleterious mutation of the unrelated gene, *egfl7*, but not morpholino knockdown, induced genetic compensation in zebrafish.[44] In support of this hypothesis, we showed that cardiac expression of *adrb2b* and *adrb3b* mRNA was increased in *adrb1*$^{-/-}$ larvae.

The role of ß3-ARs in the fish heart is enigmatic.[7] Although we observed marked bradycardia in response to the ß3-antagonist SR59230A, application of the ß3-agonist BRL 37344 was without effect. BRL 37344 was previously shown to increase heart rate in trout larvae at the same concentration.[45] It should be noted that the specificity of both of these drugs has been questioned, even in mammals,[46,47] where they may exert their effects on other ß-receptor subtypes; thus, their effects should be interpreted cautiously.

The contribution of zebrafish ß2-ARs to cardiac control also is ambiguous. Previous studies have suggested that ß2-ARs exert negative chronotropic effects, given that morpholino knockdown of *adrb2a* and *adrb2b* increased heart rate[15] and treatment with reputed ß2-specific antagonist procaterol decreased heart rate.[15,48] The latter finding is dubious given that procaterol affects other zebrafish ß-ARs non-selectively. For example, procaterol behaves as an agonist for zebrafish ß1-ARs in transfected HEK293 cells.[15] There is conflicting evidence for a positive chronotropic action of zebrafish ß2-ARs. In the current study, it was demonstrated that heart rate was reduced by treatment with a putative (but not yet pharmacologically confirmed in zebrafish) ß2-specific antagonist ICI-118551. Importantly, it was shown previously that activation of zebrafish ß2-ARs increases intracellular cAMP akin to ß1-AR stimulation.[15] In pacemaker cardiomyocytes, such an increase in cAMP would be expected to activate hyperpolarization-activated cyclic nucleotide–gated (HCN) channels, which are known to determine heart rate in larval zebrafish,[49] and increase heart rate. The possible stimulatory effect of ß2-ARs is consistent with decades of research suggesting that ß2-ARs are primarily responsible for the binding of catecholamines and positive inotropic effect on atrial and ventricular myocardium in other fish species.[25–30] Given that both positive chronotropic and positive inotropic effects largely stem from similar mechanisms dependent on cAMP accumulation, it is reasonable to predict that ß2-ARs could exert stimulatory effects in pacemaker, atrial, and ventricular cardiomyocytes alike.

This study provides mechanistic insights into a fundamental difference in adrenergic cardiac control between zebrafish and mammals.[50] In zebrafish, the ß1-AR is dispensable for tachycardia, possibly due to redundancy with other ß-ARs. With the available evidence, it is not possible to conclude whether upregulation of ß2 or ß3-ARs (or potentially other unidentified non-adrenergic receptors) are most important in mitigating the effects of *adrb1* knockout. The results of the current study also illustrate the difficult challenge of reconciling knockdown, knockout, and pharmacological approaches in studies of cardiovascular control in zebrafish.

4 | MATERIALS AND METHODS

4.1 | Experimental animals and husbandry

All experiments were carried out in accordance with animal care guidelines provided by the Canadian Council on Animal Care and with prior approval from the University of Ottawa Animal Care Committee (Protocol BL-226). Wild-type zebrafish were sourced from the in-house stock at the University of Ottawa and maintained under standard conditions[51] in 3 or 10 L tanks in a recirculating system supplied with aerated, dechloraminated City of Ottawa tap water (hereafter "system water") maintained at 28°C with a 14:10-h light–dark cycle. Fish were fed once or twice daily with a commercial zebrafish diet. Embryos (first for the development of the knockout line and later for maintenance of the line and to supply larvae for experiments) were attained by collecting small groups of 2–3 male and 2–3 female (except where stated otherwise) adult fish in sloped 2 L static tanks overnight with a perforated base insert to allow egg collection after spawning the following morning. Larval zebrafish were fed brine shrimp nauplii and a standard commercial diet from 7 dpf.

4.2 | Generation of *adrb1*$^{-/-}$ line

We aimed to delete the entire single exon of the zebrafish *adrb1* gene with the use of CRISPR/Cas9 by using guide

RNAs (gRNAs) targeting sequences both upstream and downstream of the gene (NCBI accessions: Gene ID 557194 within genomic region NC_007123.7, chromosome 12). The guide portions of four gRNAs were designed using the CHOPCHOP program[52,53] and four corresponding target (T1-4) short-guide oligos were developed (Table 1). A DNA construct for each gRNA was generated using a PCR-based (cloning-free) method with a guide constant oligo (Table 1) as described previously.[54,55] Each 50 µl PCR reaction consisted of 5 µl of 10× DreamTaq buffer (Thermo Scientific), 0.25 µl of 5 U/µl DreamTaq DNA Polymerase (Thermo Scientific), 1 µl of 10 mM dNTPs, 1 µl of 1 µM guide constant oligo, 1.25 µl of 10 µM gDNA primer pair, 40.5 µl dH_2O, and 1 µl of the 1 µM target-specific short-guide oligo. The thermal profile of the reaction was 94°C for 3 min, 40 cycles of 94°C for 40 s, 55°C for 1 min, and 72°C for 45 s, followed by 72°C for 10 min. The PCR product was purified with the GeneJet PCR purification kit (Thermo Scientific) following the manufacturer's protocol. gRNAs were synthesized using a HiScribe T7 high-yield RNA synthesis kit (New England Biolabs, Ipswich, MA) according to the manufacturer's instructions, followed by RNA purification with ethanol precipitation. sgRNA size and quality were verified by gel electrophoresis and concentrations measured using a NanoDrop 1000 spectrophotometer (ThermoFisher, Waltham, MA, USA).

A schematic step-by-step overview of the protocol used to generate the *adrb1*$^{-/-}$ line is provided in Figure S1. Microinjections were performed in 1-cell stage embryos as described previously.[56] The injection solution comprised of the following: 40 ng/µl for each sgRNA, 20 ng/µl Cas9 protein (New England BioLabs, Ipswich, MA, USA), and 0.1% Phenol Red (Sigma, Burlington, MA, USA).

The primary injected embryos were raised to sexual maturity (i.e. F0 population) and were then individually outcrossed with naïve wild-types of the opposite sex to identify founder fish carrying a germline deletion of *adrb1*. Juveniles (~60 dpf) from these crosses were genotyped with screening primers F1 and R1 (Table 1). DNA was extracted from fin clips (collected following brief anesthesia in buffered tricaine mesylate (MS-222; Syndel Laboratories, Nanaimo, BC, Canada; 100 mg l^{-1}) in 20 µl of 50 mmol l^{-1} NaOH at 95°C for 10 min followed by neutralization with 2 µl of 1 mol l^{-1} Tris–HCl (pH 8). PCR amplification proceeded under the following conditions: 200 µmol l^{-1} dNTPs, 2.5 µl 10× DreamTaq buffer (ThermoFisher), 0.125 µl DreamTaq Hot Start DNA polymerase (ThermoFisher), and 5 µl of DNA digest, with a temperature cycle of 94°C for 3 min, and 35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 45 s, and a final 72°C step for 10 min. This amplified a ~300 bp fragment in fish carrying a whole-exon deletion of *adrb1*. A single founder was identified that reliably produced embryos (~15% fertilized eggs) carrying the *adrb1* deletion when crossed with a wild-type. Sanger sequencing (Genome Quebec, McGill University, Montreal, Canada) of the PCR product and alignment with the wild-type genomic sequence (Supplementary Material) confirmed a 2462 bp deletion encompassing the entire single exon (Figure 1A). Fourteen F1 heterozygotes (*adrb1*$^{+/-}$) containing identical deletions were then raised to sexual maturity and in-crossed to generate an F2 generation.

TABLE 1 Oligonucleotides used for the generation of CRISPR guide RNAs or as screening primers

Primer/oligo name	Sequence (5′-3′)
CRISPR/Cas9 gRNA synthesis	
T1	GCG TAA TAC GAC TCA CTA TAG **GCG TAA AGT AAA ACC CGA AG**G TTT TAG AGC TAG AAA TAG
T2	GCG TAA TAC GAC TCA CTA TA**G GAG CCA AGA GCT TGT TTT G**GT TTT AGA GCT AGA AAT AGC
T3	GCG TAA TAC GAC TCA CTA TA**G GTT TTC AGT TGG TTC AAC G**GT TTT AGA GCT AGA AAT AGC
T4	GCG TAA TAC GAC TCA CTA TAG **GTT TTC AGT TGG TTC AAC GC**G TTT TAG AGC TAG AAA TAG
Guide constant	AAG CAC CGA CTC GGT GCC ACT TTT TCA AGT TGA TAA CGG ACT AGC CTT ATT TTA ACT TGC TAT TTC TAG CTC TAA AAC
gDNA primer 1 (F)	GCG TAA TAC GAC TCA CTA TAG
gDNA primer 2 (R)	AAA GCA CCG ACT CGG TGC CAC
Genomic DNA screening	
Screening F1	TAA AGC TTC CCA CGT TCA TGG
Screening R1	CTG GAA GCC AAT GAA GGA AAG

Note: Bolded text represents specific guide portion of target (T1-4) short-guide oligos.

For screening of the F2 generation (fin clipping at ~60 dpf), we used Phire Tissue Direct PCR Master Mix kit (Thermo Scientific), according to the manufacturer's instructions. The thermal profile of the reaction was 98°C for 5 min followed by 40 cycles of 98°C for 5 s, 62°C for 5 s, 72°C for 1 min, and finally 72°C for 5 min. The enhanced polymerase of the Phire Hot Start II DNA Polymerase (compared with Taq polymerase) allowed not only amplification of the ~300 bp mutant band but additionally the ~3000 bp amplicon of the wild-type gene (Figure 1B). We confirmed that DNA extracted from naïve wild-type fish exhibited the ~3000 bp amplicon only and known F1 heterozygotes exhibited both the mutant (~300 bp) and wild-type (~3000 bp) amplicons. Screening of the F2 generation allowed us to identify and separate *adrb1*$^{-/-}$, *adrb1*$^{+/-}$, and *adrb1*$^{+/+}$ individuals at 60–90 dpf.

4.3 | Screening of F1 heterozygous crosses for the Mendelian ratio of offspring

In addition to using F1 heterozygotes (*adrb1*$^{+/-}$) to form a stable F2 *adrb1*$^{-/-}$ line, we performed a dedicated experiment to record the ratio of *adrb1*$^{-/-}$, *adrb1*$^{+/-}$, and *adrb1*$^{+/+}$ individuals in the offspring of F1 incrosses. Four separate F1 incrosses, each consisting of one adult male and one adult female, were performed and their offspring were raised separately. All of the fish in this experiment were terminally sampled at 7 and 28 dpf with MS-222 overdose (300 mg l^{-1}) because it is difficult to ensure full recovery after the invasive fin clipping procedure in sub-juveniles. Individual larvae were screened using the Phire Tissue Direct PCR Master Mix kit as described above.

4.4 | Larval heart rate measurements

For all of the experiments on larval heart rates, gene expression, and respirometry, we compared F3 *adrb1*$^{-/-}$ and *adrb1*$^{+/+}$ "cousins" generated from incrossing the screened *adrb1*$^{-/-}$ or *adrb1*$^{+/+}$ F2 populations (Figure S1). By using *adrb1*$^{+/+}$ siblings as breeders, we ensured that genetic background was controlled (Zimmer et al., 2019[57]).

Except where described otherwise (hypercapnia experiment), heart rates were measured optically in zebrafish larvae as described previously[19] using a USB microscope (Firefly GT805; Firefly Global Belmont, MA, USA), connected to a personal computer, at a resolution of 640x480 pixels and a frame rate of 32.8 frames per second. Individual larvae, maintained in a holding vessel in a water bath at 28°C, were gently but rapidly pipetted into capillary tubes and video acquisition commenced immediately. This method has previously been shown to allow the measurement of a "routine" heart rate in larval zebrafish.[58]

4.4.1 | Routine heart rate across development

We first measured routine (unanaesthetised) heart rates in *adrb1*$^{-/-}$ and *adrb1*$^{+/+}$ larvae from 2 to 21 dpf. At 2 and 3 dpf the larvae were manually dechorionated and allowed at least 30 min to recover. Measurements could not be made after 21 dpf as the heart became too difficult to observe reliably due to reduced transparency of the cuticle. Each larva was measured at only one age (non-repeated measure design) to avoid risks of habituation.

4.4.2 | Heart rate responses to adrenergic receptor antagonists and agonists

The protocols that investigated the cardiac response to exogenously applied adrenergic receptor agonists and antagonists solely employed 7 dpf larvae, an age at which previous studies have revealed a robust chronotropic response to isoproterenol.[39] To facilitate measurements, the larvae were lightly sedated with 50 μg ml^{-1} MS-222[59] during the drug exposures (15 min ± antagonist followed by 10 min ± agonist in continued presence of antagonist) and subsequent heart rate measurement. Larvae were first isolated in open top 1.5 ml centrifuge tubes (holding vessel) containing 1 ml of system water containing the anesthetic.

The first protocol investigated the effects of 15 min pretreatment with general ß-receptor blockers propranolol hydrochloride (P0884 Sigma-Aldrich Canada) or sotalol hydrochloride (S0278 Sigma-Aldrich Canada) followed by 10 min treatment with one of three adrenergic receptor agonists: isoproterenol bitartrate (I2760 Sigma-Aldrich Canada), adrenaline bitartrate (E4375 Sigma-Aldrich Canada), and noradrenaline bitartrate (A0937 Sigma-Aldrich Canada). Controls were also run with no antagonist and/or agonist. All drugs were used at 100 μM to be consistent with extensive previous work in zebrafish larvae.[15,39,60] When administering pharmacological agents in the external water, the final internal concentration reaching cardiac receptors is unknown, and at present it represents an unsurmountable challenge to reliably measure circulating (internal) concentrations empirically given the small body size and minute blood volumes of larval zebrafish. Nevertheless, because the dermis of larval fish is thin and highly vascularized,[61] and provides a relatively large surface area for diffusion, this method provides a convenient and reliable route to administer the drugs.[62] It certainly would not be expected that drug penetration would be different in *adrb1*$^{-/-}$ and *adrb1*$^{+/+}$ larvae, so thus remains a valid treatment for the purposes of

our study. The drugs were dissolved in dH_2O at a stock concentration of 10 mM and 10 µl was added to the 1 ml system water in the holding chamber.

For the second protocol, larvae were pre-treated for 15 min in the absence or presence of a ß-AR sub-type-specific antagonist; atenolol (β1 antagonist, 100 µM[23]; A7655 Sigma-Aldrich Canada), ICI-118551 hydrocholoride (β2 antagonist, 100 µM[63]; I127 Sigma-Aldrich Canada), or SR59230A (β3 antagonist, 10 µM[45]; S8688 Sigma-Aldrich Canada). Owing to the low water solubility of atenolol and SR59230A, all of these antagonists were dissolved in dimethylsulfoxide (DMSO, Sigma-Aldrich Canada) to make a 10 mM stock, or 1 mM for SR59230A, and 10 µl of the stock solution was added to the 1 ml system water in the holding vessel. To account for the effects of the vehicle, a series of experiments was conducted with DMSO only, although it has also previously been shown that 1% DMSO has little effect on heart rate in zebrafish larvae.[64] After the 15 min incubation, isoproteronol (100 µM final concentration provided as 10 µl of 10 mM stock in dH_2O to a final volume of 1 ml) was added to half of the larvae. Isoproteronol treatment, in the continued presence of the antagonist where applicable, was again maintained for 10 min before heart rate was measured.[15]

As we observed strong effects of ß3 adrenergic receptor blockade, we also studied the effects of 10 min treatment with 100 µM ß3 adrenergic receptor agonist BRL 37344 (B169, Sigma-Aldrich Canada). This agonist was dissolved in DMSO to a stock concentration of 20 mM and 5 µl was added to the 1 ml holding vessel. Parallel experiments were conducted with the same volume of DMSO (final concentration 0.5%) alone.

4.4.3 | The effect of hypercapnia on larval heart rate

To provide consistency with comparable previous work,[23] this experiment used a different approach to measure heart rate. FIve or six dpf larvae were enclosed in a glass capillary tube, closed at each end with mesh, connected to a reservoir to provide a gravity-fed flow-through (1 ml min^{-1}) of system water containing MS-222 (50 µg ml^{-1}) to attain light sedation. The temperature of the flow-through water was maintained at 28–29°C, measured at the level of the capillary tube. The heart was observed and recorded with an iPhone SE (Apple, Cupertino, CA, USA) mounted onto the eyepiece of a dissection microscope (Zeiss Discovery V8; Zeiss, Oberkochen, Germany). Larvae were allowed to rest for 10 min in normocapnic system water before the experiment commenced. Heart rate was measured every 2.5 min with 10 s videos. Larvae were first exposed to normocapnic water for 3 min, with measurements taken after 30 s and 2.5 min. They were then exposed to pre-equilibrated hypercapnic system water (1% CO_2 in air) for 10 min and measurements commenced immediately upon exposure to hypercapnia and at 2.5 min intervals thereafter. At the end of the 10 min hypercapnia exposure, the procedure was repeated to reinstate normocapnia for 5 min. Heart rates were analyzed in ImageJ and LabChart as described previously.[19] Time-matched parallel controls were run with larvae maintained in normocapnic water.

4.5 | Effects of adrenergic stimulation on isolated heart

Hearts from adult (3 month post-fertilization) *adrb1*$^{+/+}$ and *adrb1*$^{-/-}$ zebrafish were harvested to allow the direct effect of adrenergic stimulation to be studied in the spontaneously beating heart, as described previously.[19] Briefly, isolated hearts were video recorded, allowing heart rate to be measured, in a 5 ml Petri dish in physiological saline (mM: NaCl, 150; KCl, 2.5; $MgSO_4$, 1.5; NaH_2PO_4, 0.4; glucose, 10; HEPES, 10; $CaCl_2$, 1; pH set to 7.7 with NaOH) at 28°C before and 5 min after isoproterenol bitartrate (final concentration of 1 µM) treatment.

4.6 | Gene expression (qPCR)

Real-time PCR was performed to assess the expression of *adrb* genes in the heart of 7 dpf *adrb1*$^{+/+}$ and *adrb1*$^{-/-}$ zebrafish. Larvae were euthanized by chilling in an ice bath and transferred onto a microscope slide. Using a micro-scalpel, two perpendicular excisions were made to excise the heart, one following the orobranchial cavity just beneath the brain and the other perpendicular to this excision immediately behind of the heart and in front of the yolk sac. Hearts were pooled and homogenized (20 hearts per sample) using a hand-held homogenizer in 0.5 ml Trizol (15 596 018 Invitrogen). RNA was extracted from these homogenates following the manufacturer's protocol. RNA concentration was determined using a NanoDrop™ 2000 spectrophotometer (Thermo Scientific). RNA was treated with DNaseI (18 068 015 Invitrogen) prior to cDNA synthesis from 0.5 µg total RNA using a high-capacity cDNA reverse transcription kit with RNase inhibitor (Applied Biosystems, Cat#: 4374967). Real-time PCR was performed in 20 µl reactions containing 10 µl of SsoFast EvaGreen supermix (1725201 Bio-Rad), 5 µl of 2 µM forward and reverse primer pairs (Table 2), and 5 µl of cDNA template (0.5 µl reverse transcribed cDNA added to 4.5 µl of ddH_2O). Controls (no template added) were run in every assay, and non-reverse transcribed samples were run for every primer pair. Reaction efficiency for all

TABLE 2 Primers used for real time quantitative PCR

Gene	Orientation	Sequence
eef1α1l1	F	CGTTGAGAAGAAAATCGGTG
	R	CCAGTCCTTAAGTAGAGTGC
actβ1	F	CCCCTTGTTCACAATAACCT
	R	CCCACATAGGAGTCTTTCTG
gapdh	F	CAACCAAATCAGGCATAATGG
	R	AATCAAGGTCAATGAATGGGT
adrb1[a]	F	GCTGGGAATAATCATGGGAA
	R	GCTCCTTATCCACCACTTG
adrb2a[a]	F	GTCACGCTATCCTAACGTCA
	R	ATTCCTCTTTCGCCAAGTTC
adrb2b[a]	F	AAGCCTTTGAACCAAGATG
	R	GCCTTTCCAAATATGTCCTG
adrb3a[a]	F	GTTTCTCATTGCCACACGA
	R	ACTCTTCCTCTTTGCTGTCA
adrb3b[a]	F	GACTCTTGTGAAATTCCTGAAG
	R	GACTGAAGATGCCCATGATAA

Note: All sequences listed 5′ to 3′ with the reverse primer sequences listed as the reverse compliment of the gene sequence.

[a]Primer sequences obtained from Ref. [21].

primer pairs was between 90% and 110%. Relative expression of all *adrb* genes was normalized to the geometric mean of *actβ1*, *eef1α1l1*, and *gapdh* ($Ct_{adrb1+/+}$ = 16.993, $Ct_{adrb1-/-}$ = 16.995 [Ct, cycle threshold]) serving as reference genes[65] and then expressed relative to the expression level of *adrb1* in the *adrb1*$^{+/+}$ group.

4.7 | Respirometry

Routine metabolic rate (RMR) and critical oxygen tension (P_{crit}) were measured in 7 dpf *adrb1*$^{+/+}$ and *adrb1*$^{-/-}$ larvae using closed system respirometry. Larvae were placed into individual 80 µl respirometry wells of a 24-well glass microplate (Loligo Systems, Viborg, Denmark) fitted with O_2 sensor spots. Each trial consisted of 11 *adrb1*$^{+/+}$ and 11 *adrb1*$^{-/-}$ larvae, along with 2 blanks for background control. The microplate was sealed with adhesive plate seals (AB0580, ThermoFisher Scientific, Mississauga, Canada) and maintained at 28°C using a water bath. The sealed microplate and water bath were attached to an O_2 fluorescence sensor (SDR SensorDish Reader, PreSens, Regensburg, Germany), and PO_2 levels were measured until they plateaued, at which point the experiment was terminated. Larval fish weights were obtained from the same batches of larvae used for the respirometry experiment. Fish (n = 20) were pooled into a single 20 µm cell strainer (pluriSelect, San Diego, USA) and excess water was removed by centrifugation (500×g). Weights were measured using an analytical balance and six pooled weights were obtained for each genotype. No differences were observed for the weights between *adrb1*$^{+/+}$ (0.161 ± 0.005 mg per larva) and *adrb1*$^{-/-}$ (0.161 ± 0.008 mg per larva) larvae, and thus the average weight for both genotypes was used for downstream $\dot{M}O_2$ (µmol g^{-1} h^{-1}) calculation. $\dot{M}O_2$ and P_{crit} data were analyzed using the calc_rate() and pcrit() functions from the respR package[66] in R (https://www.r-project.org/). $\dot{M}O_2$ was obtained from the linear decline of PO_2 during the course of the experiment, beginning from ~15 min into the experiment until ~2 h. P_{crit} data are reported as the value obtained from the segmented method in the analysis. In addition, P_{crit} regression lines were inspected "blindly" for quality control, and only those deemed to be valid traces were retained. Traces that were excluded either did not have a stable oxyregulatory phase in the PO_2 versus $\dot{M}O_2$ curve to fit a tightly fitted line or results obtained from the broken-stick method[67] and the segmented method[68] using the pcrit() function differed substantially.

4.8 | Statistical analyses

Statistical analysis and graph construction was completed in GraphPad Prism (v. 9.3.1; GraphPad Software Inc., San Diego, CA, USA). Chi-square tests were used to compare observed ratios of *adrb1*$^{-/-}$, *adrb1*$^{+/-}$, and *adrb1*$^{+/+}$ from the F1 incross with expected Mendelian ratio (25%, 50%, 25%) at 7 dpf, 28 dpf, and the age groups pooled. A two-way analysis of variance (ANOVA) was used to compare heart rates in *adrb1*$^{-/-}$ and *adrb1*$^{+/+}$ larvae (genotype) and age from 2 to 21 dpf. Three-way ANOVAs were used to analyze the effects of genotype, agonist (adrenaline, noradrenaline, or isoproterenol), and antagonist (propranolol and sotalol) for pharmacological protocol 1, and genotype, agonist (isoproterenol), and antagonist (DMSO vehicle, atenolol, ICI-118551, or SR59230A) for pharmacological protocol 2. A two-way ANOVA was used to analyze the effects of genotype and BRL-37344. A repeated-measures two-way ANOVA was used to analyze the effects of hypercapnia over time and genotype. A repeated-measures two-way ANOVA was used to analyze the effects of isoproterenol treatment and genotype on in vitro isolated hearts from adult *adrb1*$^{-/-}$ and *adrb1*$^{+/+}$ zebrafish, the body masses of which were compared with an unpaired *t*-test. Unpaired *t*-tests were used to compare the expression of each *adrb* gene, oxygen consumption, and P_{crit} in *adrb1*$^{-/-}$ and *adrb1*$^{+/+}$ larvae. Data are presented as means ± standard error of the mean (SEM).

ACKNOWLEDGMENTS

We thank the University of Ottawa Aquatics facility for zebrafish care and Hong Meng Yew for assistance with the genomic screening protocol. We also acknowledge the advice of Dr Marc Ekker in generating the knockout line. Steve F. Perry received funding from the Natural Sciences and Engineering Research Council of Canada (NSERC; RGPIN 2017-05545) and William Joyce received funding from the Novo Nordisk Foundation (NNF19OC0055842).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on Figshare at https://doi.org/10.6084/m9.figshare.19524898.v1

ORCID

William Joyce https://orcid.org/0000-0002-3782-1641

REFERENCES

1. Aris-Brosou S, Chen X, Perry SF, Moon TW. Timing of the functional diversification of alpha- and beta-adrenoceptors in fish and other vertebrates. *Ann N Y Acad Sci*. 2009;1163:343-347.
2. Zavala K, Vandewege MW, Hoffmann FG, Opazo JC. Evolution of the β-adrenoreceptors in vertebrates. *Gen Comp Endocrinol*. 2017;240:129-137.
3. Furchgott RF. The pharmacological differentiation of adrenergic receptors. *Ann N Y Acad Sci*. 1967;139(3):553-570.
4. Lands AM, Arnold A, Mcauliff JP, Luduena FP, Brown TG. Differentiation of receptor systems activated by sympathomimetic amines. *Nature*. 1967;214(5088):597-598.
5. Nergårdh A, Boréus LO, Naglo AS. Characterization of the adrenergic beta-receptor in the urinary bladder of man and cat. *Acta Pharmacol Toxicol (Copenh)*. 1977;40(1):14-21.
6. Bristow MR. Treatment of chronic heart failure with β-adrenergic receptor antagonists. *Circ Res*. 2011;109(10):1176-1194.
7. Imbrogno S, Gattuso A, Mazza R, Angelone T, Cerra MC. β3-AR and the vertebrate heart: a comparative view. *Acta Physiol*. 2015;214(2):158-175.
8. Lohse MJ, Engelhardt S, Eschenhagen T. What is the role of beta-adrenergic signaling in heart failure? *Circ Res*. 2003;93(10):896-906.
9. Myagmar B-E, Flynn JM, Cowley PM, et al. Adrenergic receptors in individual ventricular myocytes: the beta-1 and alpha-1b are in all cells, the alpha-1a is in a subpopulation, and the beta-2 and beta-3 are mostly absent. *Circ Res*. 2017;120(7):1103-1115.
10. Billman GE, Castillo LC, Hensley J, Hohl CM, Altschuld RA. β2-adrenergic receptor antagonists protect against ventricular fibrillation. *Circulation*. 1997;96(6):1914-1922.
11. Bristow MR, Ginsburg R, Umans V, et al. Beta 1- and beta 2-adrenergic-receptor subpopulations in nonfailing and failing human ventricular myocardium: coupling of both receptor subtypes to muscle contraction and selective beta 1-receptor down-regulation in heart failure. *Circ Res*. 1986;59(3):297-309.
12. Barbuti A, Terragni B, Brioschi C, DiFrancesco D. Localization of f-channels to caveolae mediates specific beta2-adrenergic receptor modulation of rate in sinoatrial myocytes. *J Mol Cell Cardiol*. 2007;42(1):71-78.
13. Greene D, Kang S, Kosenko A, Hoshi N. Adrenergic regulation of HCN4 channel requires protein association with β2-adrenergic receptor. *J Biol Chem*. 2012;287(28):23690-23697.
14. Rodefeld MD, Beau SL, Schuessler RB, Boineau JP, Saffitz JE. Beta-adrenergic and muscarinic cholinergic receptor densities in the human sinoatrial node: identification of a high beta 2-adrenergic receptor density. *J Cardiovasc Electrophysiol*. 1996;7(11):1039-1049.
15. Steele SL, Yang X, Debiais-Thibaud M, et al. In vivo and in vitro assessment of cardiac -adrenergic receptors in larval zebrafish (*Danio rerio*). *J Exp Biol*. 2011;214(9):1445-1457.
16. Stoyek MR, Quinn TA, Croll RP, Smith FM. Zebrafish heart as a model to study the integrative autonomic control of pacemaker function. *Am J Physiol Heart Circ Physiol*. 2016;311(3):H676-H688.
17. Kossack M, Hein S, Juergensen L, et al. Induction of cardiac dysfunction in developing and adult zebrafish by chronic isoproterenol stimulation. *J Mol Cell Cardiol*. 2017;108:95-105.
18. Margiotta-Casaluci L, Owen SF, Rand-Weaver M, Winter MJ. Testing the translational power of the zebrafish: an interspecies analysis of responses to cardiovascular drugs. *Front Pharmacol*. 2019;10:893.
19. Joyce W, Perry SF. Hif-1α is not required for the development of cardiac adrenergic control in zebrafish (*Danio rerio*). *J Exp Zool Part Ecol Integr Physiol*. 2021;335(7):623-631.
20. Maciag M, Wnorowski A, Bednarz K, Plazinska A. Evaluation of β-adrenergic ligands for development of pharmacological heart failure and transparency models in zebrafish. *Toxicol Appl Pharmacol*. 2022;434:115812.
21. Wang Z, Nishimura Y, Shimada Y, et al. Zebrafish beta-adrenergic receptor mRNA expression and control of pigmentation. *Gene*. 2009;446(1):18-27.
22. Konrad A, Teufel AI, Grahnen JA, Liberles DA. Toward a general model for the evolutionary dynamics of gene duplicates. *Genome Biol Evol*. 2011;3:1197-1209.
23. Miller S, Pollack J, Bradshaw J, Kumai Y, Perry SF. Cardiac responses to hypercapnia in larval zebrafish (*Danio rerio*): the links between CO_2 chemoreception, catecholamines and carbonic anhydrase. *J Exp Biol*. 2014;217(19):3569-3578.
24. Hein SJ, Lehmann LH, Kossack M, et al. Advanced echocardiography in adult zebrafish reveals delayed recovery of heart function after myocardial cryoinjury. *PLoS One*. 2015;10(4):e0122665.
25. Ask JA. Comparative aspects of adrenergic receptors in the hearts of lower vertebrates. *Comp Biochem Physiol A*. 1983;76(3):543-552.
26. Ask JA, Stene-Larsen G, Helle KB. Atrial ß2-adrenoceptors in the trout. *J Comp Physiol B*. 1980;139(2):109-115.
27. Gamperl AK, Wilkinson M, Boutilier RG. β-Adrenoreceptors in the trout (oncorhynchus mykiss) heart: characterization, quantification, and effects of repeated catecholamine exposure. *Gen Comp Endocrinol*. 1994;95(2):259-272.
28. Shiels HA. Cardiomyocyte morphology and physiology. In: Gamperl AK, Gillis TE, Farrell AP, Brauner CJ, eds. *Fish Physiology*. Academic Press; 2017:55-98.
29. Stene-Larsen G. Comparative aspects of cardiac adrenoceptors: Characterization of the β2-adrenoceptor as a common "adrenaline"-receptor in vertebrate hearts. *Comp Biochem Physiol Part C Comp Pharmacol*. 1981;70(1):1-12.

30. Temma K, Hirata T, Kitazawa T, Kondo H, Katano Y. Are beta-adrenergic receptors in ventricular muscles of carp heart (*Cyprinus carpio*) mostly the beta-2 type? *Comp Biochem Physiol C*. 1986;83(2):261-263.
31. Zimmer AM, Pan YK, Chandrapalan T, Kwong RWM, Perry SF. Loss-of-function approaches in comparative physiology: is there a future for knockdown experiments in the era of genome editing? *J Exp Biol*. 2019;222(7):jeb175737.
32. Lee S, Schwinger RHG, Brixius K. Genetically changed mice with chronic deficiency or overexpression of the beta-adrenoceptors--what can we learn for the therapy of heart failure? *Pflugers Arch*. 2008;455(5):767-774.
33. Rohrer DK, Desai KH, Jasper JR, et al. Targeted disruption of the mouse beta1-adrenergic receptor gene: developmental and cardiovascular effects. *Proc Natl Acad Sci*. 1996;93(14):7375-7380.
34. MacDonald EA, Stoyek MR, Rose RA, Quinn TA. Intrinsic regulation of sinoatrial node function and the zebrafish as a model of stretch effects on pacemaking. *Prog Biophys Mol Biol*. 2017;130:198-211.
35. Hughes MC, Zimmer AM, Perry SF. Role of internal convection in respiratory gas transfer and aerobic metabolism in larval zebrafish (*Danio rerio*). *Am J Physiol-Regul Integr Comp Physiol*. 2019;316(3):R255-R264.
36. Fick A. Über die Messung des Blutquantums in den Herzventrikeln. *Sitzungsberichte Physiol Ges Zu Würzbg*. 1870;2:1-16.
37. Wang T, Joyce W, Hicks JW. Similitude in the cardiorespiratory responses to exercise across vertebrates. *Curr Opin Physiol*. 2019;10:137-145.
38. De Luca E, Zaccaria GM, Hadhoud M, et al. ZebraBeat: a flexible platform for the analysis of the cardiac rate in zebrafish embryos. *Sci Rep*. 2014;4(1):4898.
39. Schwerte T, Prem C, Mairösl A, Pelster B. Development of the sympatho-vagal balance in the cardiovascular system in zebrafish (*Danio rerio*) characterized by power spectrum and classical signal analysis. *J Exp Biol*. 2006;209(6):1093-1100.
40. Burggren WW, Dubansky B, Bautista NM. Cardiovascular development in embryonic and larval fishes. In: Gamperl AK, Gillis TE, Farrell AP, Brauner CJ, eds. *Fish Physiology*. Academic Press; 2017:107-184.
41. Gaur H, Pullaguri N, Nema S, Purushothaman S, Bhargava Y, Bhargava A. ZebraPace: an open-source method for cardiac-rhythm estimation in untethered zebrafish larvae. *Zebrafish*. 2018;15(3):254-262.
42. Steele SL, Ekker M, Perry SF. Interactive effects of development and hypoxia on catecholamine synthesis and cardiac function in zebrafish (*Danio rerio*). *J Comp Physiol B*. 2011;181:527-538. doi:10.1007/s00360-010-0544-0
43. Joyce W, Wang T. What determines systemic blood flow in vertebrates? *J Exp Biol*. 2020;223(4):jeb215335.
44. Rossi A, Kontarakis Z, Gerri C, et al. Genetic compensation induced by deleterious mutations but not gene knockdowns. *Nature*. 2015;524(7564):230-233.
45. Petersen LH, Needham SL, Burleson ML, Overturf MD, Huggett DB. Involvement of β3-adrenergic receptors in in vivo cardiovascular regulation in rainbow trout (*Oncorhynchus mykiss*). *Comp Biochem Physiol A Mol Integr Physiol*. 2013;164(2):291-300.
46. Michel MC, Ochodnicky P, Summers RJ. Tissue functions mediated by β3-adrenoceptors—findings and challenges. *Naunyn Schmiedebergs Arch Pharmacol*. 2010;382(2):103-108.
47. Schena G, Caplan MJ. Everything you always wanted to know about β3-ar * (* but were afraid to ask). *Cell*. 2019;8(4):357.
48. Steele SL, Lo KHA, Li VWT, Cheng SH, Ekker M, Perry SF. Loss of M2 muscarinic receptor function inhibits development of hypoxic bradycardia and alters cardiac β-adrenergic sensitivity in larval zebrafish (*Danio rerio*). *Am J Physiol Regul Integr Comp Physiol*. 2009;297(2):R412-R420.
49. von der Heyde B, Emmanouilidou A, Mazzaferro E, et al. Translating GWAS-identified loci for cardiac rhythm and rate using an in vivo image- and CRISPR/Cas9-based approach. *Sci Rep*. 2020;10(1):11831.
50. Joyce W, Wang T. Regulation of heart rate in vertebrates during hypoxia: A comparative overview. *Acta Physiol*. 2022;234(3):e13779.
51. Westerfield M. *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio Rerio)*. University of Oregon Press; 2000.
52. Labun K, Montague TG, Gagnon JA, Thyme SB, Valen E. CHOPCHOP v2: a web tool for the next generation of CRISPR genome engineering. *Nucleic Acids Res*. 2016;44(W1):W272-W276.
53. Montague TG, Cruz JM, Gagnon JA, Church GM, Valen E. CHOPCHOP: a CRISPR/Cas9 and TALEN web tool for genome editing. *Nucleic Acids Res*. 2014;42(Web Server issue):W401-W407.
54. Talbot JC, Amacher SL. A streamlined crispr pipeline to reliably generate zebrafish frameshifting alleles. *Zebrafish*. 2014;11(6):583-585.
55. Zimmer AM, Mandic M, Yew HM, et al. Use of a carbonic anhydrase Ca17a knockout to investigate mechanisms of ion uptake in zebrafish (*Danio rerio*). *Am J Physiol Regul Integr Comp Physiol*. 2021;320(1):R55-R68.
56. Merhi R, Kalyn M, Zhu-Pawlowsky A, Ekker M. Loss of parla function results in inactivity, olfactory impairment, and dopamine neuron loss in zebrafish. *Biomedicine*. 2021;9(2):205.
57. Zimmer AM, Do J, Szederkenyi K, et al. Use of gene knockout to examine serotonergic control of ion uptake in zebrafish reveals the importance of controlling for genetic background: a cautionary tale. *Comp Biochem Physiol A Mol Integr Physiol*. 2019;238:110558.
58. Gore M, Burggren W. Cardiac and metabolic physiology of early larval zebrafish (*Danio rerio*) reflects parental swimming stamina. *Front Physiol*. 2012;3. doi:10.3389/fphys.2012.00035
59. Tzaneva V, Perry SF. Evidence for a role of heme oxygenase-1 in the control of cardiac function in zebrafish (*Danio rerio*) larvae exposed to hypoxia. *J Exp Biol*. 2016;219(10):1563-1571.
60. van Opbergen CJM, Koopman CD, Kok BJM, et al. Optogenetic sensors in the zebrafish heart: a novel in vivo electrophysiological tool to study cardiac arrhythmogenesis. *Theranostics*. 2018;8(17):4750-4764.
61. Chávez MN, Aedo G, Fierro FA, Allende ML, Egaña JT. Zebrafish as an emerging model organism to study angiogenesis in development and regeneration. *Front Physiol*. 2016;7: . https://www.frontiersin.org/article/10.3389/fphys.2016.00056
62. Langheinrich U. Zebrafish: a new model on the pharmaceutical catwalk. *BioEssays News Rev Mol Cell Dev Biol*. 2003;25(9):904-912.

63. Kumai Y, Ward MAR, Perry SF. β-Adrenergic regulation of Na+ uptake by larval zebrafish *Danio rerio* in acidic and ion-poor environments. *Am J Physiol Regul Integr Comp Physiol*. 2012;303(10):R1031-R1041.
64. Hallare A, Nagel K, Köhler H-R, Triebskorn R. Comparative embryotoxicity and proteotoxicity of three carrier solvents to zebrafish (*Danio rerio*) embryos. *Ecotoxicol Environ Saf*. 2006;63(3):378-388.
65. McCurley AT, Callard GV. Characterization of housekeeping genes in zebrafish: male-female differences and effects of tissue type, developmental stage and chemical treatment. *BMC Mol Biol*. 2008;9:102.
66. Harianto J, Carey N, Byrne M. respR—An R package for the manipulation and analysis of respirometry data. *Methods Ecol Evol*. 2019;10(6):912-920.
67. Yeager DP, Ultsch GR. Physiological regulation and conformation: a basic program for the determination of critical points. *Physiol Zool*. 1989;62(4):888-907.
68. Muggeo VMR. Estimating regression models with unknown break-points. *Stat Med*. 2003;22(19):3055-3071.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Joyce W, Pan YK, Garvey K, Saxena V, Perry SF. Regulation of heart rate following genetic deletion of the ß1 adrenergic receptor in larval zebrafish. *Acta Physiol*. 2022;235:e13849. doi: 10.1111/apha.13849