# The role of carbonic anhydrase in cardiorespiratory responses to $CO_2$ in zebrafish (*Danio rerio)*

**Emma Kunert, B.Sc.**

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University of Ottawa

## Abstract

Adaptation to environmental fluctuations, through sensing and appropriate physiological responses, is crucial to homeostasis. Neuroepithelial cells (NECs) are putative chemoreceptors resembling mammalian Type I (glomus) cells. They have been shown to respond *in vitro* to changes in $O_2$, $CO_2$, $NH_3$ and pH. Cytosolic carbonic anhydrase (Ca17a) is thought to be involved in $CO_2$ sensing owing to its presence in NECs. A mutant line of zebrafish (*Danio rerio*) lacking functional Ca17a was generated using CRISPR/Cas9 technology and used to assess the role of Ca17a in initiating the cardiorespiratory responses to elevated $CO_2$ (hypercapnia). Unfortunately, the homozygous knockout mutants (*ca17a*$^{-/-}$) did not survive longer than ~12-14 days post fertilization (dpf), restricting experiments to early developmental stages (4-8 dpf). Changes in ventilation ($f_V$) and cardiac ($f_H$) frequency in response to hypercapnia (1% $CO_2$) in wild type (*ca17a*$^{+/+}$), heterozygous (*ca17a*$^{+/-}$) and *ca17a*$^{-/-}$ fish were used to investigate Ca17a-dependent $CO_2$ sensing and downstream signalling. Wild type fish exhibited hyperventilation during hypercapnia as indicated by an increase in $f_V$. In the *ca17a*$^{-/-}$ fish, the hyperventilatory response was attenuated markedly, but only at 8 dpf. Hypercapnic tachycardia was observed for all genotypes and did not appear to be influenced by the absence of Ca17a. Interestingly, *ca17a*$^{-/-}$ fish exhibited a significantly reduced resting $f_H$. This effect of knockout became more pronounced as the fish aged. Anesthesia did not contribute to the decreased $f_H$ in the *ca17a*$^{-/-}$ fish, nor did changes in cardiac adrenergic or cholinergic tone, which were probed using propranolol (β-adrenergic receptor blocker) or atropine (muscarinic receptor blocker). The decrease in resting $f_H$ was prevented ("rescued") when *ca17a*$^{-/-}$ embryos were injected with *ca17a* mRNA. Collectively, the results of this thesis support a role for Ca17a in promoting hyperventilation

during hypercapnia in larval zebrafish and suggest a previously unrecognized role for Ca17a in determining resting heart rate.

## Résumé

L'adaptation aux variations environnementales, par la détection et les réponses physiologiques appropriées, est cruciale pour l'homéostasie. Les cellules neuroépithéliales (NECs) sont des chémorécepteurs putatifs qui ressemblent dans les cellules glomérulaires des mammifères de Type I. Il a été démontré qu'elles répondent *in vitro* aux changements d'$O_2$, de $CO_2$, de $NH_3$, et de pH. L'anhydrase carbonique cytosolique (Ca17a) est impliquée dans la détection du CO2 en raison de sa présence dans les NECs. Une lignée mutante de poisson-zèbre (Danio rerio) manquantle Ca17a fonctionnelle a été générée en utilisant la technologie de CRISPR/Cas9 et a été utilisée pour évaluer le rôle du Ca17a dans l'initiation des réponses cardiorespiratoires à une élévation du CO2 (hypercapnie). Malheureusement, les mutants homozygoties avec le Ca17a non-fonctionnelle (*ca17a*$^{-/-}$) n'ont pas survécu plus que ~12-14 jours après la fécondation (dpf), limitant les expériences aux premiers stades de développement (4-8 dpf). Les changements de la fréquence respiratoire ($f_V$) et de la fréquence cardiaque ($f_H$) en réponse à l'hypercapnie dans les poissons du type naturel (*ca17a*$^{+/+}$), hétérozygotes (*ca17a*$^{+/-}$), et *ca17a*$^{-/-}$ ont été utilisés pour étudier la détection de $CO_2$ dépendent du Ca17a et la signalisation en aval. Les poissons *ca17a*$^{+/+}$ ont démontré une réponse hyperventilatoire pendant l'hypercapnie, qui est indiquée par une augmentation dans $f_V$. Dans les poissons *ca17a*$^{-/-}$, la réponse hyperventilatoire a été nettement atténuée, mais seulement à 8 dpf. La tachycardie hypercapnique a été observée pour tous les génotypes et ne semble pas être influencée par l'absence du Ca17a. Une observation intéressante était que les poissions *ca17a*$^{-/-}$ présentaient une $f_H$ au repos considérablement réduit. Cet effet de la mutation devenait plus prononcé à mesure que le poisson vieillissait. L'anesthésie n'a pas contribué à la diminution $f_H$ chez les poissons *ca17a*$^{-/-}$, ni les changements du tonus adrénergique ou cholinergique cardiaque, qui ont été sondés à l'aide du propranolol (un bloqueur des récepteurs β-adrénergiques) ou l'atropine (un bloqueur des récepteurs muscariniques). La

diminution de la $f_H$ au repos a été empêché (« sauvé ») lorsque les embryons *ca17a*$^{-/-}$ ont été injectés avec de l'ARNm ca17a. Collectivement, les résultats de cette thèse soutiennent lerôle de Ca17a dans la promotion de l'hyperventilation pendant l'hypercapnie chez les larves de poisson-zèbre et suggèrent un rôle non reconnu dans la détermination du $f_H$ au repos.

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| | |
|---|---|
| 5-HT | 5-Hydroxytryptamine / serotonin |
| ACh | Acetylcholine |
| ACTZ | Acetazolamide |
| ANOVA | Analysis of variance |
| ATP | Adenosine triphosphate |
| bp | Base pair |
| BSA | Bovine serum albumin |
| CA | Carbonic anhydrase |
| Cas9 | CRISPR associated protein 9 |
| CCAC | Canadian Council for Animal Care |
| ChAT | choline acetyltransferase |
| CRISPR | Clustered regularly interspaced short palindromic repeats |
| DCV | Dense core vesicles |
| DNA | Deoxyribonucleic acid |
| dpf | Days post fertilization |
| dNTP | Deoxynucleotide |
| EDTA | Ethylenediametertraacetic acid |
| $f_H$ | Cardiac frequency |
| $f_V$ | Ventilation frequency |
| hpf | Hours post fertilization |
| IHC | Immunohistochemistry |
| $K_{2P}$ | Two pore domain potassium channel |
| LOF | Loss of function |
| mRNA | Messenger RNA |
| MS-222 | Ethyl 3-aminobenzoate methansulfonate salt |
| $N$ | Sample size |
| NEC | Neuroepithelial cell |
| PAM | Protospacer-adjacent motif |
| PBS | Phosphate buffered saline |
| PBS-T | Phosphate buffered saline – Tween |
| PCR | Polymerase chain reaction |
| PFA | Paraformaldehyde |
| RBC | Red blood cell |
| RIPA | Radioimmunoprecipitation assay |
| RM | Repeated measures |
| RNA | Ribonucleic acid |
| SEM | Standard error of the mean |
| SDS-PAGE | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| sgRNA | Single guide ribonucleic acid |
| TALK | TWIK-related alkaline activated $K^+$ |
| TASK | TWIK-related acid-sensitive $K^+$ |
| TRIS | 2-amino-2-(hydroxymethyl)propane-1,3-diol |
| TH | Tyrosine hydroxylase |

| | |
|---|---|
| VAChT | Vesicular acetylcholine transporter |

# 1 Introduction

## 1.1 *Overview*

The ability of fishes to sense and respond appropriately to environmental stressors is paramount to their survival. A common environmental stressor that may fluctuate temporally or spatially in aquatic habitats is carbon dioxide ($CO_2$). Elevated $CO_2$ in natural ecosystems has been a cause for concern for many years. By the end of the century, $CO_2$ levels in oceans are projected to reach 0.7 mm Hg, decreasing pH by 0.4 (McNeil and Matsumoto, 2019); the $CO_2$ levels in freshwater (FW) systems are also expected to increase (reviewed by McNeil and Matsumoto, 2019). An increased partial pressure of carbon dioxide ($PCO_2$) is termed hypercapnia and is deleterious to fish. As a weak acid, $CO_2$ forms $H^+$ and $HCO_3^-$ when hydrated, and therefore elicits respiratory acidosis (a decrease in blood pH associated with a rise in blood $PCO_2$). In turn, respiratory acidosis can impede cellular processes that are pH-dependent. Fish, therefore, have developed an array of physiological responses aimed at minimising or correcting the detrimental effects of hypercapnia and regulating the respiratory acidosis. In FW fishes, the gill and kidney are major sites of ionic and acid-base regulation. The gill is the major site of metabolic pH compensation, where the excretion of acid equivalents occurs during hypercapnia-induced acidosis (reviewed by Perry and Gilmour, 2006; Perry et al., 2003). While originally thought to have a minor role in acid-base regulation, the kidney is now known to be essential in this process (reviewed by Perry et al., 2003). The kidney has a marked $HCO_3^-$ reabsorption capacity, possibly mirroring that of the mammalian proximal tubule, which is responsible for 80-90% of renal $HCO_3^-$ reabsorption (reviewed by Perry et al., 2003). Therefore, while it is the gill that excretes large amounts of acid, the kidney is fundamental in acid-base regulation to allow

fish to regulate respiratory acidosis by accumulating plasma $HCO_3^-$ levels (reviewed by Perry et al., 2003).

Other physiological responses that occur during hypercapnia are cardiorespiratory adjustments (Crocker et al., 2000; Miller et al., 2014; Perry, 1999; reviewed by Gilmour, 2001; Milsom, 2012; Perry and Abdallah, 2012; Perry and Gilmour, 2002) that also may assist in mitigating the detrimental effects of hypercapnia. It has been recognized for decades that specific sensory cells termed chemoreceptors are able to sense the change of gas levels in the fish's environment (Burleson and Smatresk, 1990; McKendry and Perry, 2001; McKendry et al., 2001; Perry and Reid, 2002; Perry et al., 1999; Sundin et al., 1999). Based on research conducted over the past two decades, a specialized group of chemoreceptors termed neuroepithelial cells (NECs) specifically detect $O_2$ and $CO_2$ (Jonz et al., 2004; Qin et al., 2010). Although there is no direct evidence, there are indirect data linking NEC activation to the cardiorespiratory responses to hypercapnia (Koudrina et al., 2020; Miller et al., 2014). Indeed, there is general consensus that NECs function *in vivo* as polymodal chemoreceptors that initiate a suite of physiological responses during hypoxia or hypercapnia (reviewed by Gilmour and Perry, 2007; Jonz et al., 2015; Perry and Abdallah, 2012).

The mechanisms whereby NECs detect and elicit a response to $CO_2$ are currently being investigated. However, it was determined that a group of potassium ($K^+$) channels known as TASK (TWIK-related acid-sensitive $K^+$) channels are involved in the process, by responding to a drop in pH, which signals the release of neurotransmitters (Koudrina et al. 2020; Peña-Münzenmayer et al., 2014). It was also observed that NECs contain the enzyme carbonic anhydrase 17a (Ca17a; previously referred to as CAc or CA2-like a; see Esbaugh et al., 2005; Lin et al., 2008) which prompted research into its role in chemoreception (Abdallah et al., 2015;

Miller et al., 2014; Qin et al., 2010). In these previous studies, the approach was to monitor the responses of fish or NECs after pharmacological inhibition of CA activity in adults, or knockdown of Ca17a in larvae using antisense morpholinos. To date, no studies have assessed the role of CA in chemoreception using a gene knockout approach. Thus, the objective of the present study was to evaluate the involvement of Ca17a in $CO_2$ sensing by comparing wild type zebrafish (*Danio rerio*) to zebrafish lacking Ca17a (*ca17a*$^{-/-}$; Zimmer et al., 2020).

### 1.2 *Cardiorespiratory responses to $CO_2$*

Hypercapnia triggers several physiological responses in fish, including respiratory and cardiac adjustments. Documented respiratory responses include alterations of ventilation frequency and/or amplitude (reviewed by Gilmour, 2001), while cardiac responses include altered heart rate and blood pressure (reviewed by Gilmour and Perry, 2007). These responses may help lessen the magnitude of the acidosis resulting from increased $CO_2$.

#### 1.2.1 Respiratory responses

Hyperventilation occurs in species from every major fish group in response to hypercapnia (reviewed by Gilmour, 2001; Milsom, 2012; Perry and Abdallah, 2012). It is defined as an increase in the volume of ventilatory water flowing over the gills, brought about by an increase in ventilation frequency and/or stroke volume (amplitude; reviewed by Gilmour, 2001). While hyperventilation undoubtedly increases arterial $PO_2$ during hypoxia (reviewed by Gilmour, 2001; Perry et al., 2009a), its effect in reducing $PCO_2$ and alleviating acid-base stress during hypercapnia is less obvious. Although hyperventilation during hypercapnia cannot abolish respiratory acidosis, undoubtedly it attenuates the rise in $PCO_2$ with even small changes being physiologically significant to blood acid-base status owing to the log/linear relationship between pH and $PCO_2$ (see Perry and Abdallah, 2012).

### 1.2.2 Cardiac responses

Hypercapnia also affects the cardiovascular system in fish. Bradycardia, or a reduction in cardiac frequency ($f_H$), has been documented in several fish species (see reviews by Gilmour and Perry, 2007; Milsom, 2012). There has been some debate (see Perry and Desforges, 2006) regarding the benefit of bradycardia in aiding gas exchange. In theory, bradycardia could improve branchial gas transfer owing to lamellar recruitment and increased gas permeability via increases in mean ventral aortic pulsatility (reviewed by Gilmour and Perry, 2007; Perry and Abdallah, 2012), however the existing empirical evidence is equivocal. Bradycardia is achieved by activation of the parasympathetic nervous system via stimulation of cardiac muscarinic receptors (Holmgren, 1977; Taylor et al., 1977; reviewed by Taylor, 1992). In rainbow trout (*Oncorhynchus mykiss*), the bradycardia resulting from hypercapnia is a specific response to environmental $CO_2$ rather than to a reduction in ambient pH (Perry et al., 1999).

At the other end of the spectrum is tachycardia, which is an increase in $f_H$ resulting from stimulation of sympathetic pathways and activation of cardiac β-adrenergic receptors (Crocker et al., 2000; Miller et al., 2014). Several studies have found that adult white sturgeon (*Acipenser transmontanus*), and tench (*Tinca tinca* L.) respond to hypercapnia by increasing $f_H$ (Crocker et al., 2000; Randall and Shelton, 1963), as do larval zebrafish (Miller et al., 2014).

## 1.3 *Sensing environmental $CO_2$*

### 1.3.1 $CO_2$ chemoreceptors

Chemoreceptors are cells or clusters of cells that sense changes in the chemical composition of the external or internal environment, typically triggering responses aimed at homeostasis. For many years it was thought that fish were unable to directly sense changes in $CO_2$; rather, that responses to elevated $CO_2$ were caused indirectly by changes in blood $O_2$ status

associated with Root and Bohr effects (Perry and Gilmour, 2002). However, there is now ample evidence that fish specifically sense $CO_2$. For example, elasmobranchs, which lack a Root effect, are able to respond to changes in $CO_2$ (Heisler et al., 1988; Perry and Gilmour, 1996; Wood et al., 1990). Chemoreceptors responsible for cardiorespiratory responses are primarily found on the gill of adult fishes (reviewed by Milsom, 2002; Milsom, 2012a; Porteus et al., 2012), although there is evidence in some species that they may also reside in the orobranchial cavity, pseudobranch and skin (Florindo et al., 2006; Mckenzie et al., 1991; Regan et al., 2011). The $CO_2$-sensitive chemoreceptors appear to vary in their location on the gill depending on species, either being exclusively located on the first gill arch or on all four arches (reviewed by Milsom, 2012). Chemoreceptors that control cardiac frequency were found to be exclusively on the first gill arch of trout and tambaqui (*Colossoma macroponum*), and on all four arches of dogfish (*Squalus acanthias*), channel catfish (*Ictalurus punctatus*), traira (*Hoplias malabaricus*) and jeju (*Hoplerythrinus unitaeniatus;* Milsom, 2012). The receptors that control ventilatory frequency are found on all four gill arches of the species that have been investigated, although tambaqui shows evidence of additional extra-branchial receptors (Milsom, 2012). The $CO_2$-sensitive chemoreceptors appear to be externally oriented, sensing changes in water $PCO_2$ ($P_wCO_2$) rather than arterial blood $PCO_2$ ($P_aCO_2$; Milsom, 2012). For example, by using the CA inhibitor acetazolamide or hyperoxia treatment, it was demonstrated that increased $P_aCO_2$ without an increase in $P_wCO_2$ was ineffective at eliciting hyperventilation (Gilmour et al., 2005; McKendry and Perry, 2001).

### 1.3.2 Neuroepithelial cells

It is widely accepted that NECs are $CO_2$ chemoreceptors (reviewed by Perry and Abdallah, 2012). Dunel-Erb et al. (1982) were the first to identify NECs in fish gills and

characterized them as containing dense core vesicles (DCV; i.e. synaptic vesicles) likely storing the neurotransmitter serotonin (5-HT; Dunel-Erb et al., 1982). NECs can be identified by immunohistochemistry using antibodies against 5-HT (Dunel-Erb et al., 1982; Jonz and Nurse, 2003) and they have been identified in all fish species examined (reviewed by Perry et al., 2009a). In the majority of species, they are located on the distal portion of the gill filament, although in goldfish (*Carassium auratus*) and zebrafish they are additionally found scattered throughout the lamellae (reviewed by Perry and Tzaneva, 2016). NECs in fish are functionally analogous to the chemosensory Type I cells of the carotid body in mammals as both are innervated by the glossopharyngeal nerve (NECs are additionally innervated by the vagus nerve), both are mitochondrion rich (reviewed by Porteus et al., 2012), and both are populated with DCV (reviewed by Porteus et al., 2012). The carotid body and NECs differ in their origin; where the carotid body is neural crest-derived, NECs are derived from the endoderm and are thought to be homologous with mammalian pulmonary neuroepithelial bodies (Hockman et al., 2017). Whereas glomus cells contain several neurotransmitters including acetylcholine (ACh), catecholamines, ATP, and 5-HT, NECs in the majority of species examined appear to contain only 5-HT (reviewed by Perry and Tzaneva, 2016). In some species, NECs may additionally contain catecholamines because in channel catfish, a subset of putative chemoreceptors (believed to be branchial NECs) was found to be immunoreactive for tyrosine hydroxylase (TH), the rate limiting enzyme of catecholamine synthesis (Burleson et al., 2006). However, TH was not found in trout, goldfish or Asian stinging catfish (*Heteropneustes fossilis*; reviewed by Porteus et al., 2012). The pseudobranch of trout also has been found to be immunoreactive to TH, indicating the presence of catecholamines (Porteus et al., 2013). Additionally, vesicular acetylcholine transporter (VAChT), a marker for ACh, has been found in nerve fibres of the gill filament in

goldish and trout (Porteus et al., 2013; reviewed by Porteus et al., 2012) and NEC-like cells of the mangrove rivulus (*Kryptolebias marmoratus*; Regan et al., 2011) and adult zebrafish (Zachar et al., 2017). Additionally, colocalization of VAChT and choline acetyltransferase (ChAT) has been observed in NEC-like cells of adult zebrafish (Zachar et al., 2017). These results suggest ACh may be released at a synapse in the gills in response to gas stress.

From a functional viewpoint, there are several subtypes of NECs. One subtype is a dual sensor of $CO_2$ and $O_2$ (Qin et al., 2010), similar to Type I cells, which are sensitive to changes in $O_2$, $CO_2$ and pH (reviewed by Gonzalez et al., 1992; Gonzalez et al., 1994). Another subtype is responsive to either $O_2$ or $CO_2$ but not both (Qin et al., 2010). There is also evidence that NECs are sensitive to $NH_3$ (Zhang et al., 2011), thus establishing at least some as trimodal gas sensors.

The first evidence for chemoreceptor activity at the gill was provided by denervation experiments and employment of pharmacological agents (Burleson and Milsom, 1993; Burleson and Milsom, 1995a; Burleson and Milsom, 1995b; Burleson and Smatresk, 1990; Milsom and Brill, 1986). However, despite the ample results of *in vitro* studies demonstrating the chemosensitivity of NECs (Jonz et al., 2004) and responses to $CO_2$ (Abdallah et al., 2012; Abdallah et al., 2015; Qin et al., 2010), there is little *in vivo* evidence directly implicating them as the branchial chemoreceptors facilitating cardiorespiratory responses. The lack of evidence reflects the difficulty of performing *in situ* electrophysiological recordings or in achieving NEC loss-of-function phenotypes (reviewed by Perry and Tzaneva, 2016).

The locations of NECs are markedly different in adult and larval zebrafish, the only species where NECs have been investigated at both stages. In adults, NECs are found on the gill filament and lamellar epithelium (Jonz et al., 2004). In zebrafish and trout, the NECs are localized within the leading edge of the gill filament in close proximity to the efferent filament

artery (Dunel-Erb et al., 1982). Thus, they are well situated to monitor changes in water and/or arterial gas partial pressures (Jonz and Nurse, 2003). In larval zebrafish, however, the NECs are situated predominantly on the skin (Coccimiglio and Jonz, 2012; Jonz and Nurse, 2006). Cutaneous NECs are present as early as 24-26 h post-fertilization (hpf) and are at their highest density at 3 d post-fertilization (dpf; Coccimiglio and Jonz, 2012). The density of NECs on the skin decreases steadily as NECs begin to emerge on the developing gill (Coccimiglio and Jonz, 2012). Innervation of NECs on the gill filaments begins at 5 dpf, and by 7 dpf they are fully innervated as lamellae begin to develop (Jonz and Nurse, 2006).

### 1.3.3 Mechanism of $CO_2$ detection

It is theorized that background/leak $K^+$ channels are involved in $CO_2$ sensing by NECs (Abdallah et al., 2015; Koudrina et al., 2020; Peña-Münzenmayer et al., 2014; Qin et al., 2010). Zebrafish filament NECs *in vitro* were shown to depolarize during hypercapnia, a response that was prevented by the blockade of background $K^+$ channels with quinidine (Qin et al., 2010).

It is probable that as in Type I cells, NECs express several types of $K^+$ channels. Of these, a possible candidate for $CO_2$/pH sensing is the TASK-2 channel, which is a member of the TALK subfamily $K_{2P}$ channels. While the name 'TASK' seems to associate TASK-2 with members of the TASK subfamily (a family of acid-sensitive $K_{2P}$ channels), this channel is sensitive to pH in the alkaline range and is therefore classed in the TALK (TWIK-related alkaline activated $K^+$) subfamily (reviewed by Enyedi and Czirjak, 2010). It is a probable choice because it responds to both extracellular and intracellular pH and is deactivated by acidification (Niemeyer et al., 2010; Peña-Münzenmayer et al., 2014). In zebrafish, TASK-2 and/or TASK-2b appear to play a role in facilitating cardiorespiratory responses to hypercapnia (Koudrina et al., 2020). When either paralog was knocked down, the cardiorespiratory responses to hypercapnia

were blunted (Koudrina et al., 2020). There was no observed additive effect when both paralogs were knocked down (Koudrina et al., 2020), indicating that loss of either paralog is sufficient to impair the $CO_2$/pH sensing pathway.

TASK-1 and TASK-3 also were investigated as possible $CO_2$/pH sensors in mammals, with evidence both for and against their role. For example, although supporting evidence for their involvement in controlling ventilation was reported (reviewed by Buckler, 2015; Enyedi and Czirjak, 2010), mice that underwent single and double knockout of TASK-1 and -3 continued to responded to hypercapnia with an increase in minute ventilation (Mulkey et al., 2007). These results indicate that TASK-1 and -3 are not essential for respiratory chemosensitivity (Mulkey et al., 2007).

In addition to uncertainty surrounding the importance of $pH_i$ versus $pH_o$ on cellular signalling, there is some doubt as to whether NECs respond to $H^+$ or $CO_2$. Abdallah et al. (2015) suggested that NECs respond to $H^+$. They tested three conditions: acidic hypercapnia (5% $CO_2$, pH = 6.6), isocapnic acidosis (normocapnia, pH = 6.6), and isohydric hypercapnia (5% $CO_2$, pH = 7.6). Of the three treatments, only acidic hypercapnia and isocapnic acidosis resulted in a transient elevation of cytosolic [$Ca^{2+}$], likely arising from intracellular storage compartments (Abdallah et al., 2015). However, Qin et al. (2010) found that during isohydric hypercapnia, the hypercapnia-induced membrane depolarization persisted in the absence of intracellular acidosis. These differences may indicate that there are multiple pathways involved in the initiation of cardiorespiratory responses to hypercapnia. Peña-Münzenmayer et al. (2014) suggested that $CO_2$ itself, may exert a direct effect on TASK-2 during hypercapnia. Using a strongly buffered saline to keep $pH_i$ constant, they reported a continued (direct) effect of $CO_2$. Moreover, when they removed lysine 245 (the amino acid responsible for $pH_i$-sensitivity) in mouse TASK-2 by site-

directed mutagenesis (converting lysine into alanine), the response to hypercapnia was maintained.

The detection and response of NECs to $CO_2$ is expected by theory to be CA-dependent because of the role of CA in promoting intracellular acidification via catalysed hydration of $CO_2$ to $H^+$ and $HCO_3^-$. When the CA inhibitor acetazolamide (ACTZ) was used to reduce the rate and magnitude of internal acidification during hypercapnia (Abdallah et al., 2015), the depolarization response of zebrafish NECs was delayed and reduced in magnitude (Qin et al., 2010). These results indicate that full membrane depolarization is reliant on a reduction in intracellular pH ($pH_i$) arising from the hydration of $CO_2$ catalysed by CA. Further, Miller et al. (2014) explored the effect of CA morpholino knockdown on the cardiac response to $CO_2$ in larval zebrafish. Morphant zebrafish did not experience the increase in cardiac frequency exhibited by control fish when exposed to 0.75% $CO_2$ (Miller et al., 2014). These results suggest a role for CA in the physiological responses to hypercapnia.

### 1.4 *Carbonic anhydrase*

Carbonic anhydrases are a group of zinc-metalloenzymes found in all living organisms that catalyze the reversible hydration of $CO_2$. There are several families including α, β, and γ CA, with all vertebrates possessing αCA (Hewett-Emmett and Tashian, 1996). Among other processes, CA contributes to acid-base and ionic regulation, gas transfer, bone resorption and calcification (Lin et al., 2008; reviewed by Gilmour and Perry, 2009; Sly et al., 1995). A variety of categories of CA exist, including cytosolic, mitochondrial, membrane-bound and secreted variants (reviewed by Lindskog, 1997).

### 1.4.1 Structure and catalytic function

The catalytic activity of CA is dependent on its zinc ion, which is located in a cone-shaped cavity at the active site and is coordinated to three histidine residues (His-64, -96, and -119) (reviewed by Lindskog, 1997). The importance of this structure is apparent from CA isoforms that lack one or more of these histidine residues; these isoforms are referred to as CA-related proteins and are non-catalytic (reviewed by Lindskog, 1997). The zinc ion acts as a Lewis acid, accepting a pair of electrons (reviewed by Hoppert, 2011). Zinc-bound $OH^-$ enables the catalytic activity of the enzyme, as $OH^-$ attacks a $CO_2$ molecule, which in turn forms a metal-bound $HCO_3^-$ (reviewed by Lindskog, 1997). This ion is then displaced by another $OH^-$ ion, facilitated by the shuttling activity of His-64 in high activity-forms of CA, and the cycle can begin once again (reviewed by Lindskog, 1997). This catalytic mechanism bypasses the formation of $H_2CO_3$, which occurs in the slower, uncatalyzed reaction.

### 1.4.2 Relevant CA isoforms

In mammals, there are at least 16 isoforms of CA (reviewed by Gilmour and Perry, 2009; Supuran, 2018), including cytosolic, mitochondrial, membrane-bound or membrane-spanning, and secreted forms. Of the cytosolic variants including CA I, II, III and VII, CA II is the most abundant and is considered the "workhorse" of the CA isoforms (reviewed Gilmour and Perry, 2009). In mammals, CA is found in the cytosol of cells in tissues of most major organs including osteoclasts, brain, eye, kidney and pancreas to name but a few (reviewed Sly et al., 1995). The catalytic speed of CA II is exceptionally fast, with a hydration turnover rate of $1 \times 10^6$ $sec^{-1}$ (reviewed by Lindskog, 1997; Sly et al., 1995). The next fastest cytosolic isoform is CA VII, with a turnover rate of $4 \times 10^5$ $sec^{-1}$, followed by CA I with a turnover rate of $2 \times 10^5$ $sec^{-1}$, and finally CA III with a rate of $8 \times 10^3$ $sec^{-1}$ (reviewed by Lindskog, 1997; Sly et al., 1995).

The distribution and nomenclature of CA isoforms differ considerably in fish (Gilmour and Perry, 2009), although this conclusion is based largely on investigations restricted to zebrafish (Lin et al., 2008) and trout (Esbaugh et al., 2005). A predominant difference is that the distinct clade found in mammals of cytosolic CA I, II and III is not present in fish (Esbaugh et al., 2005). Rather, there are two distinct cytosolic CA paralogs that were first described in rainbow trout and named CAb (predominantly expressed in red blood cells; RBC) and CAc (with widespread cytosolic expression) (reviewed by Gilmour et al., 2009). In zebrafish, these same paralogs were termed zca2-like b and zca2-like a (Lin et al., 2008). More recently, these paralogs were renamed Ca17a (widespread), and Ca17b (RBC) (Ferreira-Martins et al., 2016); the latter nomenclature will be used for the remainder of the present thesis.

### 1.4.3. The possible involvement of Ca17a in $CO_2$ detection

Immunohistochemical analysis has shown that Ca17a is colocalized with a subpopulation of NECs in zebrafish (Miller et al., 2014; Qin et al., 2010), suggesting by analogy to the carotid body glomus cells (Lahiri and Forster, 2003), a role for Ca17a in the detection of and resultant physiological responses to $CO_2$. In the last decade, studies using CA inhibitors or morpholino knockdown of Ca17a have provided some support for this theory. For example, Qin et al. (2010) demonstrated that inhibition of CA activity in isolated NECs using ACTZ attenuated the magnitude and slowed the onset of $CO_2$-induced membrane depolarization. Additionally, Miller et al. (2014) reported similar inhibitory effects of ACTZ in blunting the cardiac responses of larval zebrafish to hypercapnia. Furthermore, Abdallah et al. (2015) found that during acidic hypercapnia (pH 6.6, 5% $CO_2$), gill filament NECs treated with ACTZ displayed a slower rate of acidification than untreated cells (Abdallah et al., 2015).

Acetazolamide is a non-specific inhibitor of CA and therefore inhibits all isoforms. Because Ca17a is believed to be the isoform involved in $CO_2$ sensing owing to its presence in NECs, it is important that techniques be used to specifically inhibit or remove this isoform. Miller et al. (2014) used morpholino knockdown to prevent normal Ca17a function in larval zebrafish and reported that the fish experiencing Ca17a knockdown exhibited an attenuated cardiac response to hypercapnia. A limitation of knockdown experiments is that effective reduction of function is restricted to a relatively brief period of development. For example, Miller et al (2014) noted that significant loss of CA activity after morpholino injection was reliably sustained until only 5 dpf.

### 1.5 *Objectives, hypothesis, and predictions*

Owing to the absence of isoform specificity of ACTZ as a CA inhibitor and the short duration of effective morpholino knockdown, the main objective of the present thesis was to investigate the role of Ca17a in $CO_2$ chemoreception and downstream cardiorespiratory reflexes using a line of Ca17a-knockout zebrafish (Zimmer et al., 2020). Because the knockout fish survived for only 12-14 dpf (Zimmer et al., 2020), all experiments were conducted using larvae between 2 and12 dpf. An initial series of experiments was conducted to determine the experimental levels of $CO_2$ required to elicit cardiorespiratory responses as a function of developmental age. Once the appropriate levels of hypercapnia were determined, experiments were performed to compare knockouts (*ca17a*$^{-/-}$), wild types (*ca17a*$^{+/+}$) and heterozygous fish (*ca17a*$^{+/-}$) with respect to ventilatory and cardiac responses to hypercapnia.

Because Ca17a is expressed in NECs (Abdallah et al., 2015; Miller et al., 2014; Qin et al., 2010), and based on the previous results of inhibitor/knockdown experiments (see above), it was hypothesised that Ca17a is involved in $CO_2$-mediated cardiorespiratory responses.

Therefore, it was predicted that fish lacking Ca17a would exhibit attenuated ventilatory and cardiac responses to hypercapnia compared to wild type fish.

During the analysis of $f_H$ responses, it was noted that the resting $f_H$ of *ca17a*$^{-/-}$ fish was significantly lower than those of *ca17a*$^{+/+}$ or *ca17a*$^{+/-}$ fish. This led to further questions concerning the underlying mechanism causing this notable reduction. A series of additional experiments was conducted to probe this unexplained discrepancy using pharmacological methods and an mRNA rescue experiment.

## 2 Materials and methods

### 2.1 *Zebrafish husbandry*

Adult zebrafish (*Danio rerio*) were housed in 10 L flow-through tanks at the University of Ottawa aquatics facility. Facility system water was dechloraminated city of Ottawa tap water (in mM, 0.8 $Na^+$, 0.4 $Cl^-$, 0.25 $Ca^{2+}$; pH 7.6) kept at 28ºC, and the facility photoperiod was 14:10 h light:dark. Embryos were obtained by placing 2 males and 3 females in a 2 L tank overnight. The next morning embryos were collected with a fine mesh filter and observed with a microscope to select embryos in early developmental stages (≤2 hpf). Embryos ≤2 hpf were placed into Petri dishes with E3 embryo medium (in mM; 0.33 $CaCl_2 \cdot 2H_2O$, 0.17 Cl, 0.33 $MgSO4 \cdot 6H_2O$, 5 NaCl, 0.0001% methylene blue) at a density of 30 embryos per dish. Embryos were kept in an incubator held at 28ºC; embryo medium was changed daily. Fish kept past 6 dpf were moved to 1 L static tanks and fed GEMMA 75 micro fish feed (Skretting USA, Westbrook, ME, USA) on alternate days. Procedures for animal use were in accordance with the University of Ottawa Animal Care Committee guidelines under approved protocols BL-226 and BL-2118. All procedures adhered to the guidelines of the Canadian Council for Animal Care (CCAC) for animal use in research and teaching.

### 2.2 *CRISPR/Cas9 knockout of ca17a*

The line of *ca17a* knockout zebrafish developed and validated by Zimmer et al. (2020) was used in the current experiments. In brief, a single guide RNA (sgRNA) was synthesized with the guide portion targeting a region of exon 1 (GGCGGAATGGCGACCACTG), which was upstream of PAM (protospacer-adjacent motif) GGG (guide designed using CHOPCHOP). The sgRNA was column purified (RNeasy mini kit, QIAGEN), verified by gel electrophoresis, and its final concentration was determined by spectrophotometry. Cas9 mRNA was synthesized

using an mMESSAGE mMACHINE SP6 kit (Invitrogen, Cat#: AM1340), with quality being confirmed by gel electrophoresis and concentration determined by spectrophotometry. A solution containing 150 pg $nL^{-1}$ Cas9 mRNA, 50 pg $nL^{-1}$ of sgRNA, and 0.1% phenol red in Danieau buffer was injected into embryos at the single cell stage, and fish were raised to reproductive maturity. As established previously (Zimmer et al., 2020), homozygous *ca17a* knockouts (*ca17a*$^{-/-}$) did not survive past 14 dpf making it impossible to maintain a breeding stock. Heterozygous (*ca17a*$^{+/-}$) mutants survived to maturity and therefore were used as breeders. Thus, to obtain *ca17a*$^{-/-}$ mutants for experimentation, it was necessary to screen and genotype larvae from heterozygous crosses at 3 dpf to obtain wild types (*ca17a*$^{+/+}$) and homozygous knockouts prior to experimentation. In some experiments, data were collected on individual fish that were then euthanized and genotyped by genome sequencing (see below).

Fin clipping and multiplex PCR were used to screen larval zebrafish prior to experimentation using the method of Zimmer et al. (2020), which was adapted from techniques outlined in two previous studies (Kosuta et al., 2018; Wilkinson et al., 2013). In brief, 3 dpf larvae were anesthetized with TRIS-buffered 0.3 mg $mL^{-1}$ MS-222 (ethyl-3-aminobenzoate methanesulfonate salt; Syndel Laboratories, Nanaimo, BC, Canada) and the caudal tail (distal to blood circulation) was removed using a microscalpel (Fine Science Tools, North Vancouver, BC, Canada) under a dissection microscope. After clipping, fish were rinsed with system water and placed in individual wells of a 96-well plate while awaiting screening results; they were moved into Petri dishes once results were obtained. The small portion of excised tail was smeared onto a piece of filter paper that was placed into the well of a 96-well PCR plate containing 10 µl of a 0.25:1 mixture of DNARelease Additive and Dilution Buffer from a Phire Tissue Direct PCR Master Mix kit (ThermoScientific, Burlington ON). The filter papers were digested at 98°C for 3

min. A 10 µL reaction was prepared using the digested DNA as a template for the multiplex PCR assay. Each reaction contained 1.6 µL 10X DreamTaq buffer (ThermoFisher, Waltham, MA, USA), 0.32 µL of a 10 mM dNTP stock (ThermoFisher), 0.08 µL HotStart DreamTaq Polymerase (ThermoFisher) and 0.4 µL of DNA digest. It also contained two sets of *ca17a* primers. The first set (forward 3'-CCTCCTGCTGATCACACTTAAT-5'; reverse 3'-GGCAGCCTATAGCCAAATGTA-5') was designed to amplify a 477 bp sequence from all genotypes. The second set (forward 3'-GTATCCCCAGTGGTCAGC-5' and 3'-CTGGCGGAATGGCAGTGCATTC-5') was designed to specifically amplify the *ca17a*$^{+/+}$ (147 bp) and *ca17a*$^{-/-}$ (352 bp) alleles. Temperature cycles of the reaction were 94ºC for 3 min, followed by 30 cycles of 94ºC for 30 s, 52ºC for 1 min, 72ºC for 1 min, and a final step of 72ºC for 10 min. The PCR products were imaged on 2% agarose gels stained with RedSafe (Froggabio, North York, ON, Canada).

Throughout this study, *ca17a* genotypes identified by multiplex PCR were confirmed regularly by genome sequencing using Genome Quebec Sanger sequencing services (https://www.genomequebec.com/canadian-centre-for-computational-genomics-c3g/). Genome sequencing was also used to identify *ca17a* genotypes for individual fish used without pre-screening. For genome sequencing, whole larvae after experimentation were digested in 20 µL of 50 mM NaOH at 95ºC for 10 min. A 25 µL reaction was prepared using 10 µL of digested sample as template, 2.5 µL 10X DreamTaq buffer, 0.5 µL of a 10 mM dNTP stock, 0.125 µL HotStart DreamTaq Polymerase and 0.05 µL each of the general *ca17a* forward and reverse primers (see above).

2.3 *Whole mount immunohistochemistry*

Larval zebrafish at 4 dpf were euthanized with 4 mg $mL^{-1}$ TRIS-buffered MS-222 and fixed for 24 h at 4°C in 4% paraformaldehyde (PFA) prepared in phosphate buffered saline (PBS) containing 0.1% Tween-20 (PBS-T). Following fixation, samples were dehydrated in a stepwise manner by incubating with 50 and 75% MeOH (each for 10 min) and then left to dehydrate in 100% MeOH for 24 h at -20°C. A stepwise rehydration was then performed using 100, 75 and 50% MeOH in 0.1% PBS-T in 10 min steps. Next, the fish underwent an additional antigen retrieval step that involved immersion in 1 mL of Tris-HCl for 10 min at room temperature followed by 15 min at 65°C and 5 x 5 min washes in PBS-T. Prior to incubation with primary antibody, larvae were immersed for 2 h at room temperature in a blocking solution consisting of 3% bovine serum albumin (BSA; Sigma-Aldrich, Oakville, ON, Canada) containing 0.8% Triton X-100 and 0.1% PBS-T. Using established protocols (e.g., Coccimiglio and Jonz, 2012; Miller et al., 2014; Zimmer et al., 2020), primary polyclonal antibodies against 5-HT (1:250 dilution of goat anti-5-HT; ImmunoStar, cat#: 20079) and Ca17a (1:250 dilution of rabbit anti-Ca17a produced by Genscript custom antibody service – see Miller et al. 2014) were added and the specimens were left overnight at 4°C. Larvae were rinsed 5 x 5 min with 0.1% PBS-T and then incubated with donkey anti-goat secondary antibody conjugated to Alexa Fluor 594 (Thermo Fisher, cat#: A-11058) and goat anti-rabbit secondary antibody conjugated to Alexa Fluor 488 (Thermo Fisher, cat#: A-32731) in 0.8% Triton X-100 in 0.1% PBS-T (1:500 dilutions) for 1 h in the dark. After another round of 5 x 5 min washes, the samples were mounted in depression slides with 1.5% warm agarose and VectaShield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). Larvae were imaged using an Olympus FV1000 BX61 LSM confocal microscope (Olympus Corporation, Tokyo, Japan) with continuous wave lasers emitting at 488 and 559 nm, or a Nikon

A1RsiMP confocal microscope (Nikon Instruments Inc., Tokyo, Japan) using continuous wave lasers emitting at 488 and 561 nm. Images were composed using the maximum intensity projection of Z-stacks of optical sections of 3 µm thickness.

## 2.4 *Experimental procedures*

### 2.4.1 Determining age-dependent $CO_2$ thresholds for hyperventilatory responses in wild-type (*ca17a*$^{+/+}$) larvae

Individual larvae were lightly anaesthetized in 0.05 mg mL$^{-1}$ TRIS-buffered MS-222 in system water and when immobile, were drawn into a capillary tube (1 mm internal diameter) using a peristaltic pump (ThermoFisher Scientific). A small piece of sponge was inserted into one end of the tube to act as a physical barrier that prevented the fish from exiting the tube while still allowing for the flow of anaesthetic-containing water (24.2°C after exiting the capillary tube). Constant water flow (2 mL min$^{-1}$) was provided by a pressure head and adjusted using a flow controller attached to the tubing. Fish were left for 10 min to adjust to the normocapnic control conditions (air-equilibrated water). Larvae that did not breathe at least once during this 10 min period were discarded. Each trial consisted of a 5 min normocapnic treatment (baseline), followed by a 15 min hypercapnic treatment (exposure), and finishing with 5 min of normocapnia (recovery). Rapid switching between normocapnic and hypercapnic water was facilitated by a three-way valve connecting the two water sources to the main water inflow line. During switches between normocapnic and hypercapnic water, the flow was stopped (valve closed to the downstream inflow tubing) and a 50-mL syringe connected to the valve was used to prime the main inflow tube with the appropriate water. Videos were recorded using an iPhone SE mounted to a Zeiss Discovery V8 dissection microscope.

Wild-type larvae at 4, 6 and 8 dpf were used to determine the partial pressures of $CO_2$ ($PCO_2$) required to elicit hyperventilatory responses as determined by measuring $f_V$. The levels of $CO_2$ were varied using a 2-channel gas mixer (constructed at uOttawa) that provided mixtures (2000 L $min^{-1}$) of $CO_2$ and air to equilibrate system water (at 28$^oC$) that was supplied to larvae during experiments. The levels of $CO_2$ used were 0.5 (3.75 mm Hg), 1.0 (7.5 mm Hg), 1.5 (11.3 mm Hg) and 2.0% (15.0 mm Hg). A control group was also tested, in which the fish were exposed to air-equilibrated water for the duration of the experiment which otherwise mimicked the protocol of the $CO_2$ exposures.

2.4.2 Effects of hypercapnia (1% $CO_2$; 7.5 mm Hg) on $f_V$ and heart rate ($f_H$) across genotypes

Based on the results of the preliminary experiments using various levels of $CO_2$ (see above), 1% $CO_2$ was used in all subsequent experiments designed to evaluate the age-dependent effects of hypercapnia on $f_V$ and $f_H$ in wild type (*ca17a*$^{+/+}$), heterozygous (*ca17a*$^{+/-}$), and mutant (*ca17a*$^{-/-}$) larvae.

2.4.3 A comparison of baseline $f_H$ between wild type (*ca17a*$^{+/+}$) larvae and *ca17a*$^{-/-}$ mutants

Owing to a marked difference in the baseline $f_H$ between wild types and knockouts in the larvae at 4-8 dpf, $f_H$ was surveyed more thoroughly by extending the age range of the larvae examined. Thus, a younger age group (2 dpf) and two older age groups (10 and 12 dpf) were added to the previous data set (4, 6 and 8 dpf larvae). In these additional experiments, $f_H$ was monitored for 10 min under baseline conditions.

2.4.4 Behavioural analysis of activity

The activity of 8 dpf larvae was assessed using a Zebrabox system with the tracking extension of the Zebralab software (Viewpoint Life Sciences, Montreal QC). Analysis was performed in an isolated room maintained at 28.5°C. Behavioural analysis was carried out in a

24-well plate, which was calibrated for size and background-subtracted; the detection threshold was set at 15. Larvae were given an initial 1 h acclimation in the dark before analysis commenced. Analysis began with a 10-min dark period, followed immediately by a 10-min light period. Activity and inactivity (defined as a movements at < 2 cm $s^{-1}$) parameters included counts of activity vs inactivity, distance moved (mm), and duration of movement (s), which were measured over 30-s intervals. Total duration of inactivity and total distance moved during the dark exposure were calculated from the software's output.

2.4.5 Assessing the potential interfering effects of anaesthesia

To verify that MS-222 did not cause changes in $f_H$ that might account for the bradycardia in the *ca17a*$^{-/-}$ mutants, separate groups of wild types and *ca17a*$^{-/-}$ mutants at 8 dpf were monitored with or without anaesthetic. Larvae were first placed for at least 15 min into 1.5 mL microcentrifuge tubes containing system water; in a separate group, the water contained 0.05 mg $mL^{-1}$ of TRIS-buffered MS-222. Water temperature was maintained at 24.2$^{o}$C, for consistency with previous experiments. Larvae were gently pipetted into a capillary tube (1.2 mm internal diameter), which permitted restraint with minimal stress and a 'routine' heart rate (Gore and Burggren, 2012). Heart rate measurements commenced within 10 s to avoid large changes in temperature or $O_2$, and videos were recorded for 60 s. Larvae within the capillary tube were orientated to allow a lateral view that permitted unobstructed observation of the heart. Videos were acquired at 30 frames per second with a USB microscope (Firefly GT805, Firefly Global Belmont, MA, USA) connected to a personal computer. Pulsatile change in pixel intensity, equating to $f_H$, was determined using an automated approach (see below).

.4.6 Autonomic control of heart rate

To study whether differences in resting heart rate between knockout and wild-type fish could be explained by differences in cardiac autonomic tone, larvae were incubated for 15 min with atropine (muscarinic ACh receptor antagonist) or propranolol (general β-adrenergic receptor antagonist) immediately prior to the heart rate measurement (final concentrations of $10^{-4}$ M for both, as previously used in zebrafish larvae; Schwerte et al., 2006). Videos were recorded and analyzed using the method adopted for the MS-222 experiment (see above). Assistance with these experiments, and those of section 2.4.4, was provided by post-doctoral fellow Dr. William Joyce.

2.4.7 Rescue experiments – injection of *ca17a* mRNA into *ca17a*$^{-/-}$ embryos

Assistance with these experiments was provided by Ph.D. student Yihang Pan (synthesis of Ca17a mRNA) and aquatic care core facility technician Vishal Saxena (injection of *ca17a* mRNA). A zebrafish Ca17a cDNA open reading frame (ORF) plasmid was purchased from Genscript (CloneID: ODa159791). Ca17a cDNA along with a poly(A) signal was then PCR-amplified from the plasmid with an SP6 promoter sequence included in the forward primer (Fwd: 5'- ATTTAGGTGACACTATAG AGAACCCACTGCTTACTGGC -3', Rev: 5' – GCCCCCGATTTAGAGCTTGA). The resulting PCR amplicon was gel-purified using the PureLink™ quick gel extraction kit (Invitrogen, Cat#: K210012) and served as the DNA template for *ca17a* mRNA synthesis. Capped *ca17a* mRNA was synthesized using the mMESSAGE mMACHINE™ SP6 transcription kit (Invitrogen, Cat#: AM1340) and purified using MEGAclear™ transcription clean-up kit (Invitrogen, Cat#: AM1908) following the manufacturer's protocol. Before injection, the *ca17a* mRNA was mixed with Danieau buffer and 0.01% phenol red. Embryos were microinjected with 127.6 pg capped *ca17a* mRNA at the single cell stage, and raised to 4 dpf in an incubator held at 28.5°C. At 4 dpf, larvae were used either for

measurement of $f_H$ (using the method adopted for the MS-222 experiment) or for confirmation of Ca17a protein presence. Western blots were used to confirm the presence of Ca17a and thus to judge the effectiveness of the mRNA rescue. Two control samples from pooled *ca17a*$^{+/+}$ and *ca17a*$^{-/-}$ genotypes were used, each with one fresh 4 dpf sample, and one 9 dpf sample previously confirmed to express or lack Ca17a (Zimmer et al. 2020). The controls were used to verify that *ca17a*$^{-/-}$ samples did not contain Ca17a, while *ca17a*$^{+/+}$ samples did. These were compared to *ca17a* mRNA-injected samples, in which both *ca17a*$^{+/+}$ and *ca17a*$^{-/-}$ samples were expected to express Ca17a. Pooled samples consisted of 20 larvae, which were homogenized in 200 µL of RIPA buffer (in mM; 150 NaCl, 50 Tris-Cl, 1 EDTA, 1 phenylmethanesulfonyl fluoride, 1% Triton X-100, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate) containing a protease inhibitor cocktail (Roche, Mississauga, ON, Canada). Western blotting was performed as described previously (Yew et al., 2020) using a custom rabbit polyclonal antibody against Ca17a (1:1000 dilution of a 2 mg $L^{-1}$ stock; Genscript, Piscataway, NJ, USA) as well as a rabbit polyclonal antibody against β-actin (1:4000 dilution; Sigma-Aldrich, St. Louis, MO, USA; cat#: A2066). A minor difference from Yew et al. (2020) was the use of 12% SDS-PAGE gels rather than 10%.

## 2.5 *Analytical methods*

### 2.5.1 Visual analysis of $f_V$

Ventilation frequency was analyzed using behavioural software (Boris version 7.9.7; Friard and Gamba, 2016), which enabled keyboard logging of specific "behaviours" including breathing, swimming or change of orientation. Breaths were counted by observing mouth or opercular/buccal cavity movements depending on the orientation of the fish. Over the 25-min duration of a trial, every observable breath was counted in 1-min intervals. If a fish was active (e.g., swimming) for >50% of the 1-min period, that measurement was not included in the

average. The $f_V$ for normocapnia (baseline) for an individual fish was taken to be the mean of the five 1-min intervals of the normocapnic period. The peak $f_V$ for an individual fish was taken to be the 1-min interval exhibiting the greatest $f_V$ over the 15-min period of hypercapnic exposure.

2.5.2 Visual analysis of $f_H$

Heart rate was determined manually by tallying beats using a handheld counter while viewing the videos at 0.5-1x speed. Because movement of the fish interfered with automatic detection techniques (see below), this manual method was used when fish movement was more likely. Heart rate was determined for 15-s intervals between the following time points; 2-3, 4-5, 6-7, 9-10, 12-13, 16-17, 19-20, 20-21 and 24-25 min. This approach allowed for the observation of time-related changes in $f_H$. The baseline $f_H$ for an individual fish was calculated as the mean for the two 1-min analysis intervals prior to hypercapnia. Peak $f_H$ for an individual fish during hypercapnia was determined to be the 1-min analysis period with the greatest $f_H$ from among the five 1-min analysis intervals that occurred during the hypercapnic exposure.

2.5.3 Automated analysis of $f_H$

For experiments that only required the determination of $f_H$ for short durations (i.e., < 1 min) an automated approach was preferred. The video was opened in ImageJ software (https://imagej.nih.gov/ij/index.html) and the heart was circled as a region of interest (ROI). Using the Time Series Analyzer V3, a time period of 15 s was chosen for analysis. Upon completion, a summary showing peak intensity based on change in pixelation (i.e., heart beats), was displayed and the peaks for the 15-s interval were counted.

2.5.4 Neuroepithelial cell density

Neuroepithelial cell (NEC) densities were determined for larval (4, 6 and 8 dpf) zebrafish prepared for whole mount immunohistochemistry of 5-HT. Larvae were imaged using a Nikon

A1R+ confocal microscope (Nikon Instruments, Tokyo Japan) with 561 nm emission. Images were composed using the maximum intensity projection Z-stacks of optical section of 3 µm thickness. Cells were counted using the point-to-point counting feature on ImageJ software (National Institutes of Health, Bethesda, MD, USA). Cells were counted in three distinct regions: the head, the yolk sac and the tip of the tail. Each area was measured in ImageJ and cell density was determined (cells $mm^{-2}$).

2.6 *Statistical analyses*

Data are presented as mean values ± one standard error of the mean (SEM). Effects of hypercapnic exposure on $f_V$ and $f_H$ over time were evaluated using one-way repeated measures analysis of variance (RM ANOVA). The effects of the partial pressure of $CO_2$ ($PCO_2$) on peak $f_V$ were evaluated using two-way RM ANOVA, with exposure (baseline or hypercapnia) and $PCO_2$ (0 to 2%) as factors. Similarly, effects of hypercapnia on peak $f_V$ and peak $f_H$ across Ca17a genotypes were evaluated using two-way RM ANOVA, with exposure (baseline or hypercapnia) and genotype (*ca17a*$^{+/+}$, *ca17a*$^{+/-}$, *ca17a*$^{-/-}$) as the two factors. Effects of genotype on NEC density were evaluated by ANOVA. Effects of anaesthetic, autonomic blockers, and *ca17a* mRNA rescue were evaluated using two-way ANOVA, with genotype and treatment as the two factors in each case. Holm-Sidak tests were used for multiple comparisons when significant differences were detected by ANOVA. If data failed to meet the assumptions of normality or equal variance, they were transformed prior to analysis. Where assumptions could not be met through transformation, analyses were carried out on ranked data. The level of significance for all analyses was 0.05. All statistical analyses were performed in SigmaPlot (v11.0 and 13.0; Systat Software, Inc., San Jose California USA).

# 3 Results

## 3.1 *The effects of age and $CO_2$ levels on the ventilatory response to hypercapnia in wild type (*ca17a$^{+/+}$*) zebrafish larvae*

The initial experiments were performed solely on wild types, to characterize responses to different $CO_2$ levels. The time-dependency of the ventilatory response to acute (15 min) hypercapnia is illustrated in Fig. 1. A similar biphasic pattern was observed in all age groups; the $CO_2$-evoked increases in $f_V$ occurred rapidly during exposure to hypercapnia, reaching peak values within 1-2 min of onset. After the initial spike in $f_V$, ventilation gradually diminished over exposure time despite the continued exposure to hypercapnia (Fig. 1). Although the dose- and age-dependency of the ventilatory response to $CO_2$ was apparent from the results plotted in Fig. 1, a comparison of peak $f_V$ responses was performed to enable a detailed statistical analysis. Based on peak responses, the extent of the hyperventilation during hypercapnia (as determined from peak increases in $f_V$) increased with age and percentage $CO_2$ (Fig. 2). At 4 dpf, levels of $CO_2$ above 0.5% (3.75 mm Hg) caused hyperventilation (Fig. 2A). At 6 and 8 dpf, all levels of $CO_2$ resulted in hyperventilation (Fig. 2B, C). Regardless of age, the percentage of $CO_2$ that caused a maximum increase in $f_V$ was 1.5% (11.25 mm Hg); increasing $CO_2$ to 2.0% (15.0 mm Hg) did not increase $f_V$ further. Thus, all subsequent experiments used 1% $CO_2$.

## 3.2 *The age-dependent effects of* ca17a *genotype on the ventilatory responses to 1% $CO_2$ in zebrafish larvae*

The *ca17a*$^{+/+}$ and *ca17a*$^{+/-}$ fish experienced an increased $f_V$ response to hypercapnia between 4 and 6 dpf with no further increase at 8 dpf (Figs. 3, 4). The *ca17a*$^{-/-}$ fish also exhibited an increase in the peak $f_V$ response to hypercapnia from 4 to 6 dpf, but then a decline at 8 dpf (Figs. 3, 4). The effects of Ca17a knockout on the hypercapnic ventilatory response varied with

age. At 4 dpf, the *ca17a*$^{-/-}$ fish exhibited an elevated $f_V$ during hypercapnia compared to the other genotypes (Figs. 3A, 4A). At 6 dpf, the *ca17a*$^{-/-}$ fish responded to hypercapnia similarly to the other genotypes (Figs 3B, 4B), whereas at 8 dpf, the hyperventilatory response was blunted markedly in the *ca17a*$^{-/-}$ fish (Fig. 3C, 4C).

3.3 *The age-dependent effects of* ca17a *genotype on the neuroepithelial cell density in zebrafish larvae*

Neuroepithelial cell (NEC) density was calculated for three isolated areas on the fish: head, mid body including yolk sac, and tail. The values for these areas were combined to determine total NEC density for each fish. No significant differences among genotypes for total NEC density were detected at any age (Fig. 5). Cell density appeared to decrease during development, with initial values ~300 NEC mm$^{-2}$ at 4 dpf, dropping to ~150 NEC mm$^{-2}$ by 8 dpf (Fig. 5).

3.4 *The age-dependent effects of* ca17a *genotype on the cardiac responses to 1% $CO_2$ in zebrafish larvae*

All genotypes at all age points responded to 1% $CO_2$ with an increase in $f_H$ that was transient, except for the *ca17a*$^{-/-}$ fish at 6 dpf that responded with a sustained rise in $f_H$ (Fig. 6). The increase in $f_H$ from normocapnia to hypercapnia was approximately 13-15% of resting heart rate across all ages and genotypes, although at 6 dpf, *ca17a*$^{+/+}$ fish exhibited an increase of 10% and *ca17a*$^{-/-}$, an increase of 25%.

The *ca17a*$^{-/-}$ fish displayed a lower resting $f_H$ compared to the other genotypes across all ages (Figs. 6 and 7). To further investigate this unexpected finding, the age range was extended to 2-12 dpf and baseline $f_H$ was compared between *ca17a*$^{+/+}$ and *ca17a*$^{-/-}$ fish. In *ca17a*$^{+/+}$ fish, $f_H$

was fairly consistent across development, although it increased significantly between 4 and 8 dpf (Fig. 8A). For *ca17a*$^{-/-}$ fish, $f_H$ was lower than in wild types at all ages (Fig. 8A). Although the lowest value for baseline $f_H$ was observed at 10 dpf, the $f_H$ difference between wild types and *ca17a*$^{-/-}$ fish was more-or-less constant between 6 and 12 dpf (Fig. 8A).

Activity of individual fish of all genotypes was monitored over a 10-min period under dark conditions to determine whether Ca17a knockout had a significant impact on behaviour. No significant differences were detected in either duration of inactivity (Fig. 8B) or total distance moved (Fig. 8C).

To ensure that the lower baseline $f_H$ in the knockout fish was unrelated to anesthesia, groups of 8 dpf *ca17a*$^{+/+}$ and *ca17a*$^{-/-}$ fish were treated as usual with 0.05 mg mL$^{-1}$ MS-222 and compared to groups assessed without anesthetic. The lower $f_H$ in the *ca17a*$^{-/-}$ fish was observed regardless of the presence or absence of anesthetic (Fig. 9A). To test whether the lower $f_H$ might be attributable to differences in autonomic cardiac tone between the genotypes, fish were treated with propranolol and atropine to remove adrenergic and cholinergic tone, respectively. Propranolol resulted in a reduction of $f_H$ for both *ca17a*$^{+/+}$ and *ca17a*$^{-/-}$ fish compared to an untreated group, but *ca17a*$^{-/-}$ $f_H$ remained lower than that of *ca17a*$^{+/+}$ (Fig. 9B). Atropine raised $f_H$ for both *ca17a*$^{+/+}$ and *ca17a*$^{-/-}$ fish compared to an untreated group, but again *ca17a*$^{-/-}$ $f_H$ remained lower than that of *ca17a*$^{+/+}$ fish (Fig. 9B).

To provide additional evidence that the lower baseline $f_H$ in the *ca17a*$^{-/-}$ fish was a specific result of Ca17a loss-of-function, a ‘rescue’ experiment was performed in which *ca17a*$^{+/+}$ and *ca17a*$^{-/-}$ single cell embryos were injected with *ca17a* mRNA. The $f_H$ of *ca17a*$^{-/-}$ fish injected with *ca17a* mRNA was not lower than that of *ca17a*$^{+/+}$ fish (Fig. 10A). The $f_H$ of *ca17a*$^{+/+}$ fish injected with *ca17a* mRNA did not differ from that of the control group (Fig. 10A), indicating

that the injection itself likely did not contribute to the increase observed in the ‘rescued’ *ca17a*$^{-/-}$ fish. The success of *ca17a* mRNA injection in restoring Ca17a protein was verified by western blotting; the injected *ca17a*$^{-/-}$ fish showed Ca17a expression whereas the control *ca17a*$^{-/-}$ fish did not (Fig. 10B).

Figure 1. The effect of $PCO_2$ on ventilation frequency ($f_V$) in (A) 4 dpf, (B) 6 dpf, and (C) 8 dpf larval (wild-type) zebrafish (*Danio rerio*) exposed to 15 min of hypercapnia (indicated by the dashed lines). Symbols in red indicate times at which $f_V$ was significantly different from the corresponding 5 min baseline value (A, one-way RM ANOVA, $p = 0.031$, 0.260, < 0.001, < 0.001, < 0.001 for air to 2% $CO_2$, respectively; B, $p = 0.057$ for air, < 0.001 for 0.5% $CO_2$, < 0.001 on square-root transformed data for 1% $CO_2$, and < 0.001 on ranks for 1.5 and 2% $CO_2$; C, $p = 0.337$, < 0.001 and < 0.001 for air to 1% $CO_2$, respectively, < 0.001 on ranks for 1.5 and 2% $CO_2$). Values are means ± SEM, $N = 8$.

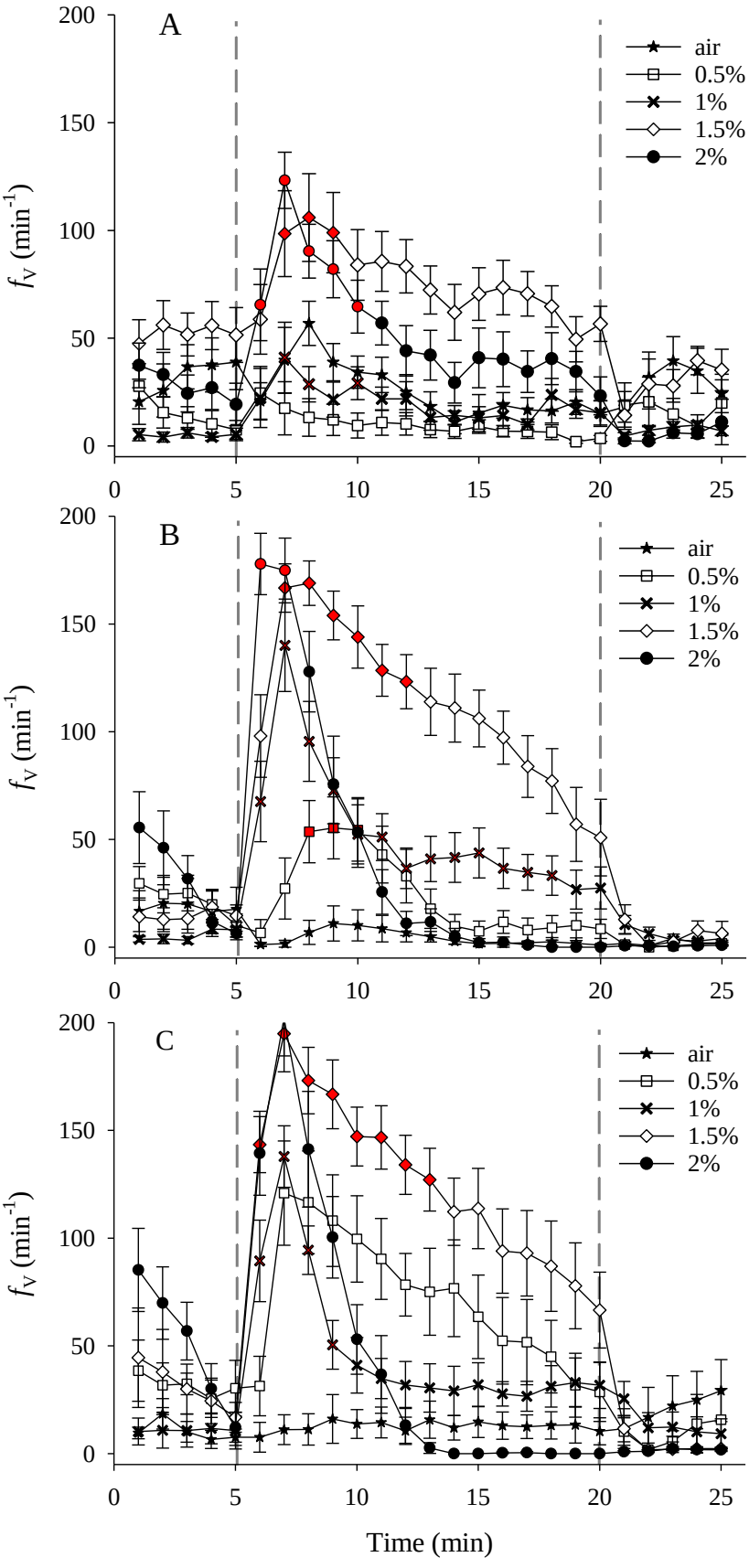

A
B
C
air
0.5%
1%
1.5%
2%
$f_V$ (min$^{-1}$)
Time (min)
0
50
100
150
200
0
5
10
15
20
25

Figure 2. The effect of $PCO_2$ on peak ventilation frequency ($f_V$) in (A) 4 dpf, (B) 6 dpf, and (C) 8 dpf larval (wild-type) zebrafish (*Danio rerio*). Solid bars present baseline (normocapnic) data averaged for each fish over the 5 min period, whereas hatched bars present peak $f_V$ during the 15 min hypercapnic exposure. In all cases, the interaction between $CO_2$ level (air to 2% $CO_2$) and exposure (baseline vs hypercapnia) was significant (2-way RM ANOVA, $p = 0.019$, $< 0.001$, on square root-transformed data, $< 0.001$, for A, B, and C, respectively). Bars that share a letter do not differ significantly from one another, with lower case letters for baseline values and uppercase letters for hypercapnia. An asterisk indicates a significant difference between baseline and hypercapnia within a $CO_2$ level. Values are means + SEM, $N = 8$.

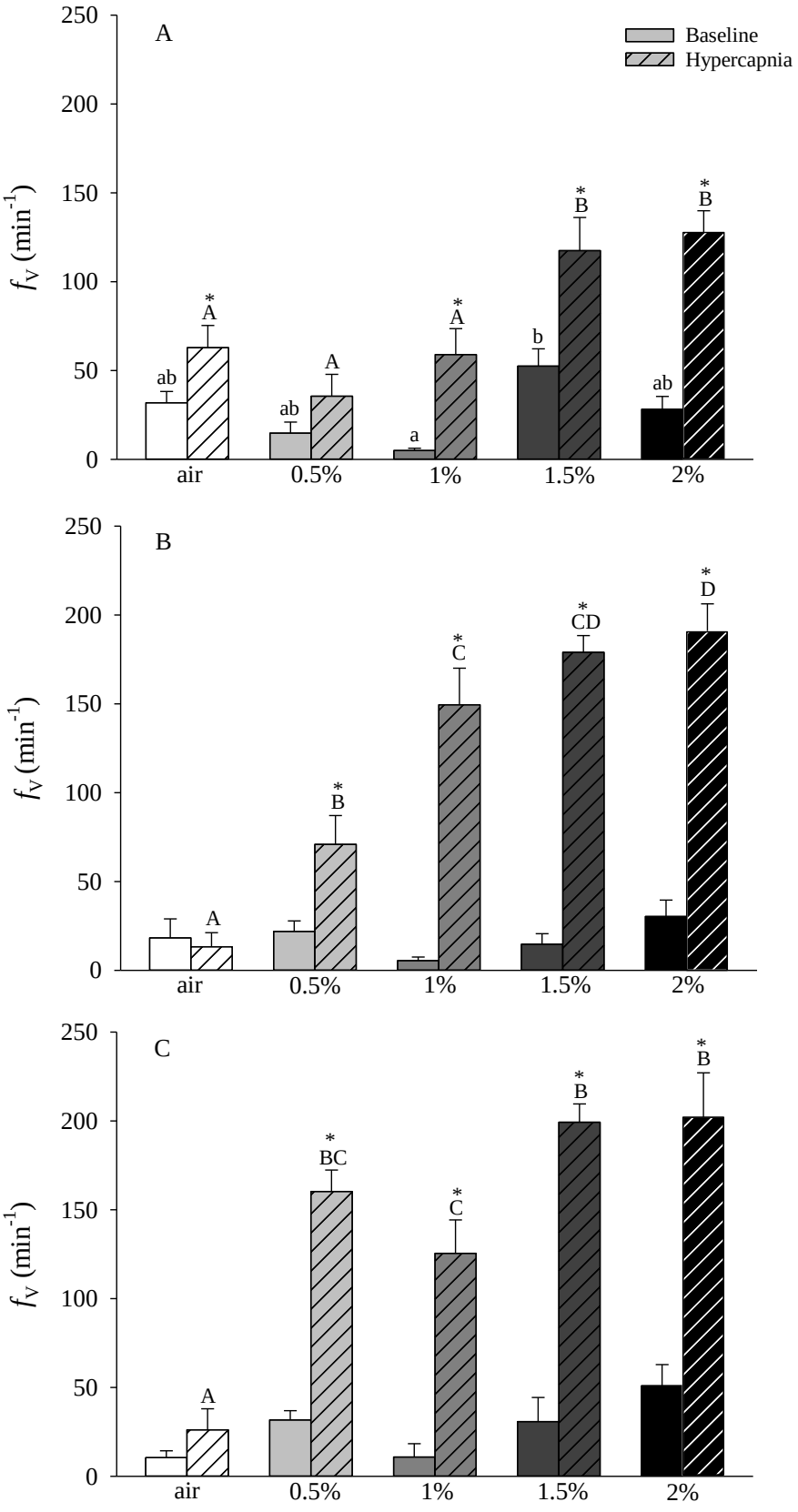

A
Baseline
Hypercapnia
$f_V$ (min$^{-1}$)
250
200
150
100
50
0
ab
*A
ab
A
a
*A
b
*B
ab
*B
air
0.5%
1%
1.5%
2%
B
A
*B
*C
*CD
*D
C
A
*BC
*C
*B
*B

Figure 3. The effect of Ca17a knockout on ventilation frequency ($f_V$) in (A) 4 dpf, (B) 6 dpf, and (C) 8 dpf larval zebrafish (*Danio rerio*) exposed to 15 min of hypercapnia (1% $CO_2$; indicated by the dashed lines). Symbols in red indicate times at which $f_V$ was significantly different from the corresponding 5 min baseline value (RM ANOVA; A, $p = 0.106$, 0.249, and <0.001 for square root-transformed data, for *ca17a*$^{+/+}$, *ca17a*$^{+/-}$, and *ca17a*$^{-/-}$, respectively; B, $p < 0.001$ for all genotypes, square-root transformed data for *ca17a*$^{+/-}$ and *ca17a*$^{-/-}$; C, $p < 0.001$ for all genotypes, ranks for *ca17a*$^{+/+}$). Values are means ± SEM, $N$ = 8-12.

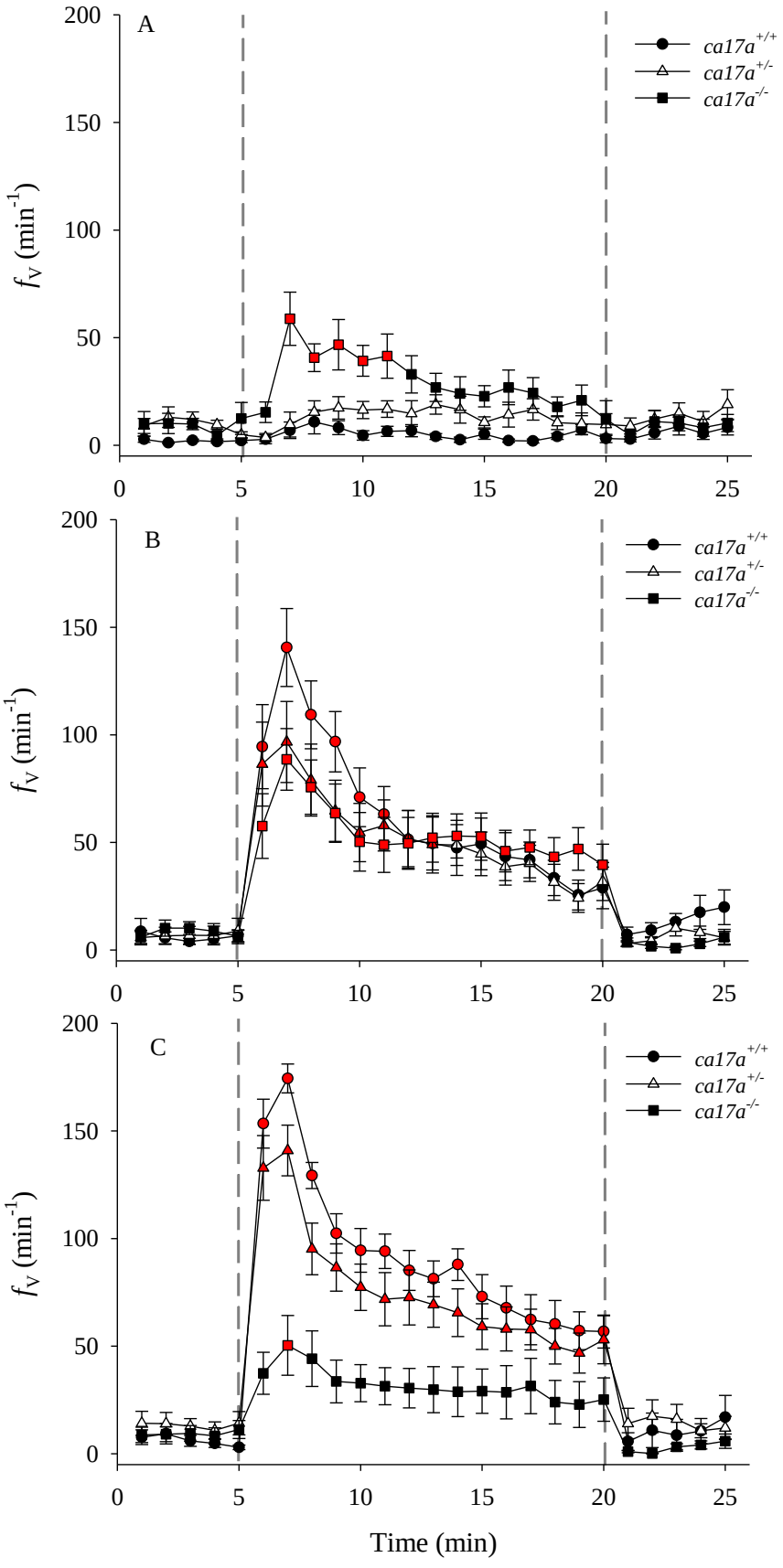

A
B
C
ca17a+/+
ca17a+/-
ca17a-/-
fV (min-1)
Time (min)
0
50
100
150
200
0
5
10
15
20
25

Figure 4. The effect of genotype on peak ventilation frequency ($f_V$) in (A) 4 dpf, (B) 6 dpf, and (C) 8 dpf larval zebrafish (*Danio rerio*). Solid bars present baseline (normocapnic) data averaged for each fish over the 5 min period, whereas hatched bars present peak $f_V$ during the 15 min hypercapnic exposure (1 % $CO_2$). Data were analyzed by two-way RM ANOVA using genotype and exposure (baseline vs hypercapnia) as the two factors. In panel A, a significant interaction was detected (square root-transformed data, $p < 0.001$). In panel B, only the effect of exposure was significant ($p_{exposure} < 0.001$, $p_{genotype} = 0.073$, $p_{exp\ x\ gen} = 0.075$). In panel C, a significant interaction was observed ($p < 0.001$). Bars that share a letter do not differ significantly from one another, and an asterisk indicates a significant difference between exposure conditions within a genotype. Values are means + SEM, $N = 10$-12.

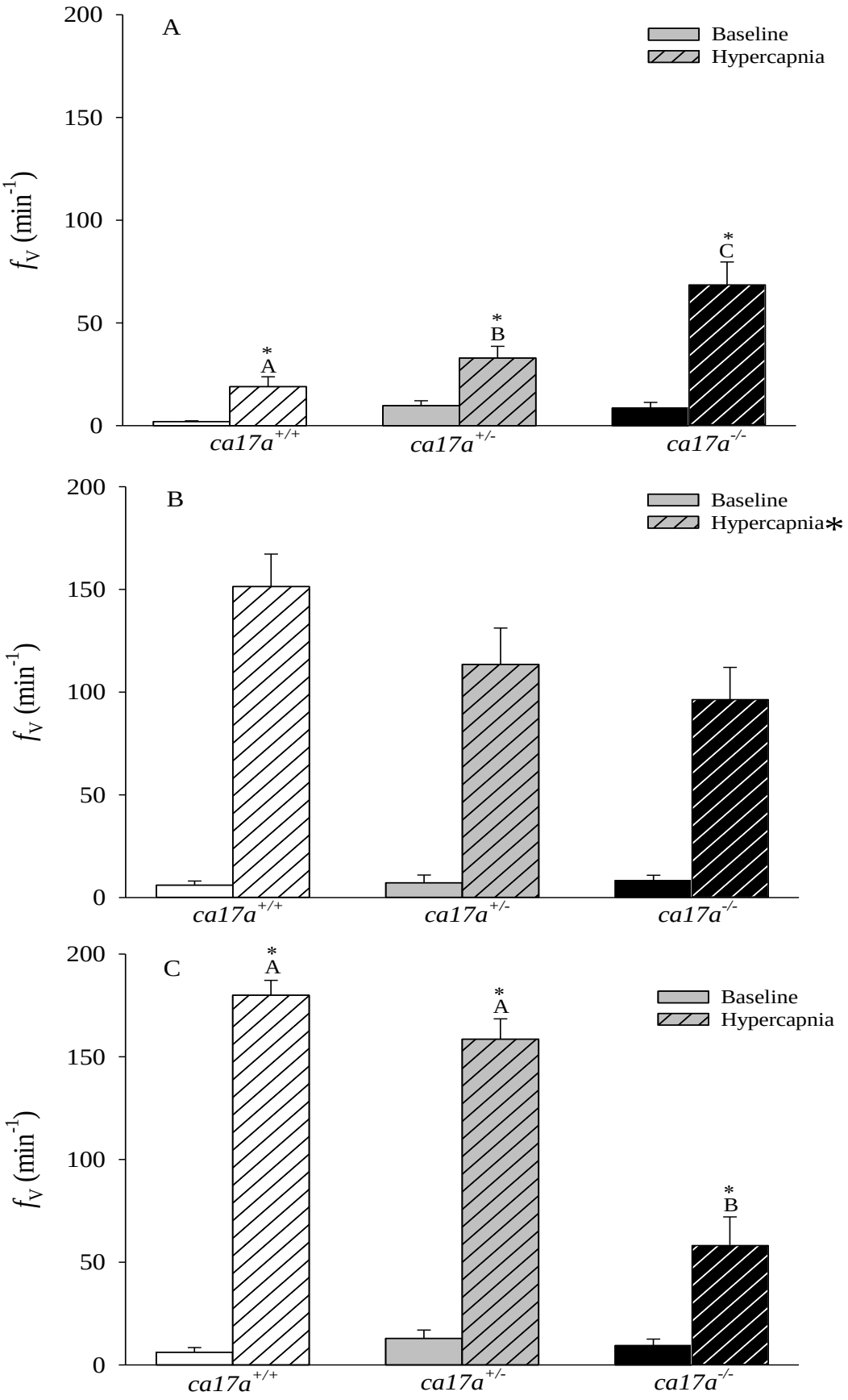

A
Baseline
Hypercapnia
200
150
100
50
0
$f_V$ (min$^{-1}$)
*
A
*
B
*
C
ca17a$^{+/+}$
ca17a$^{+/-}$
ca17a$^{-/-}$
B
Baseline
Hypercapnia*
200
150
100
50
0
$f_V$ (min$^{-1}$)
ca17a$^{+/+}$
ca17a$^{+/-}$
ca17a$^{-/-}$
C
Baseline
Hypercapnia
200
150
100
50
0
$f_V$ (min$^{-1}$)
*
A
*
A
*
B
ca17a$^{+/+}$
ca17a$^{+/-}$
ca17a$^{-/-}$

Figure 5. The effect of genotype on neuroepithelial cell (NEC) density in (A) 4 dpf, (B) 6 dpf, and (C) 8 dpf larval zebrafish (*Danio rerio*). No significant differences among genotypes were detected (one-way ANOVA, $p$ = 0.25, 0.296, and 0.062 for A, B, and C, respectively). Values are means + SEM, $N$ = 7-8.

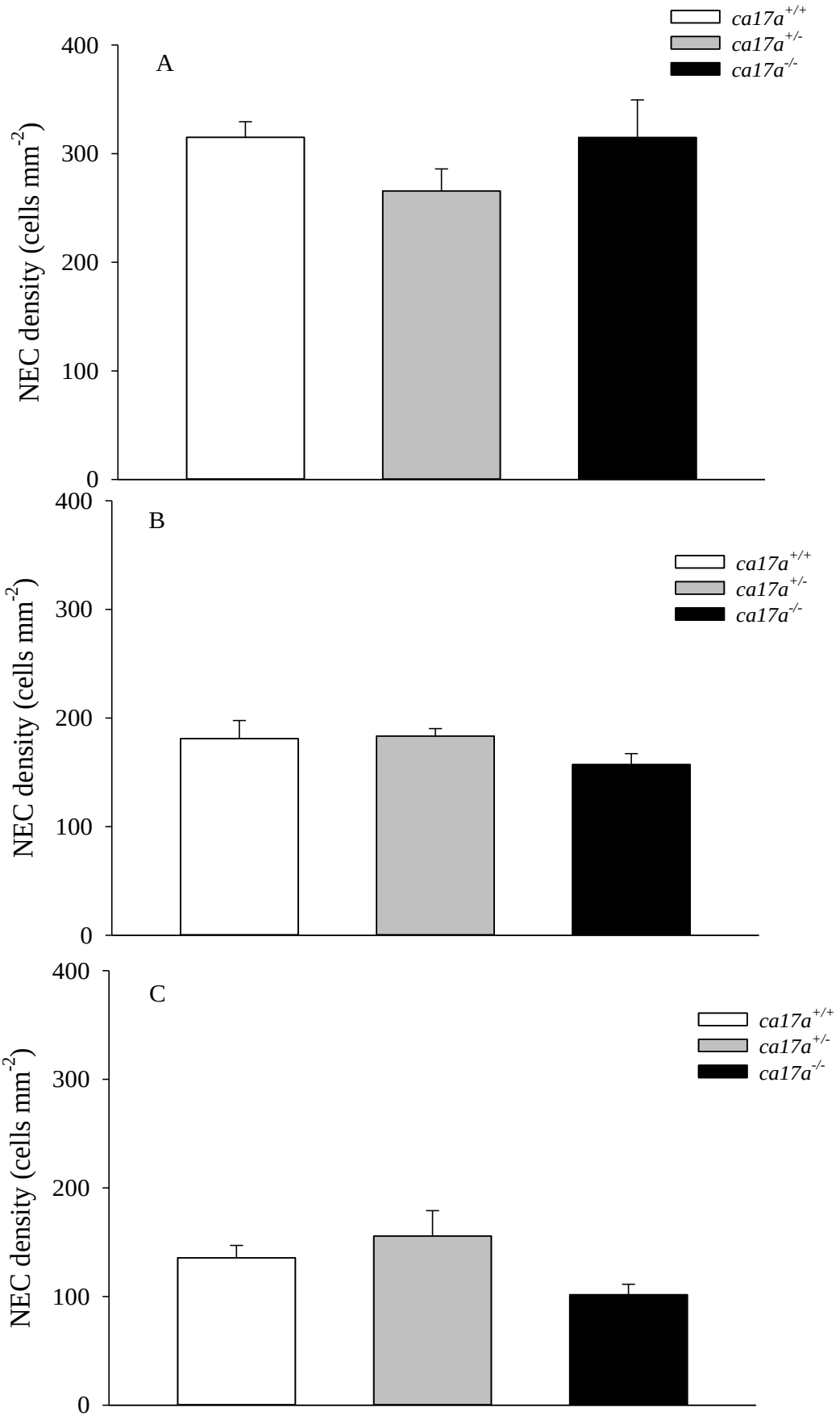

A
B
C
ca17a+/+
ca17a+/-
ca17a-/-
NEC density (cells mm-2)
400
300
200
100
0

Figure 6. The effect of Ca17a knockout on heart rate ($f_H$) in (A) 4 dpf, (B) 6 dpf, and (C) 8 dpf larval zebrafish (*Danio rerio*) exposed to 15 min of hypercapnia (1% $CO_2$; indicated by the dashed lines). Symbols in red indicate times at which $f_H$ was significantly different from the corresponding 5 min baseline value (RM ANOVA; A, $p < 0.001$ for all genotypes, on square root-transformed data for *ca17a*$^{+/-}$; B, $p = 0.131$ for *ca17a*$^{+/+}$ and $p < 0.001$ for *ca17a*$^{+/-}$ and *ca17a*$^{-/-}$; C, $p = 0.003$, 0.04 and 0.063, for *ca17a*$^{+/+}$, *ca17a*$^{+/-}$, and *ca17a*$^{-/-}$, respectively). Values are means ± SEM, $N = 10$.

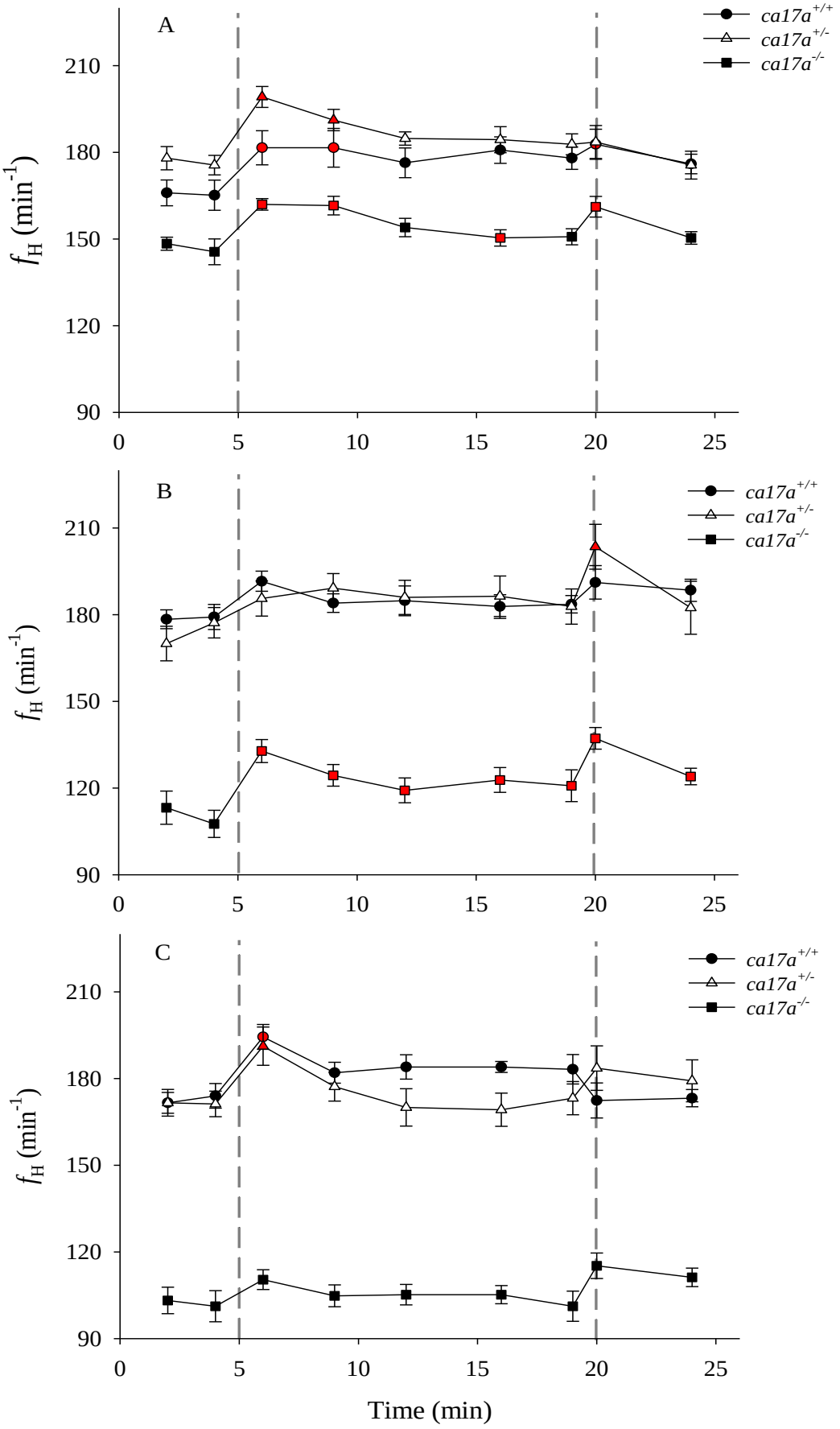

A
B
C
ca17a+/+
ca17a+/-
ca17a-/-
fH (min-1)
210
180
150
120
90
0
5
10
15
20
25
Time (min)

Figure 7. The effect of Ca17a knockout on peak heart rate ($f_H$) in (A) 4 dpf, (B) 6 dpf, and (C) 8 dpf larval zebrafish (*Danio rerio*). Solid bars present baseline (normocapnic) data averaged for each fish over the 5 min period, whereas hatched bars present peak $f_H$ during the 15 min hypercapnic exposure (1% $CO_2$). Data were analyzed by two-way RM ANOVA using genotype and exposure (baseline vs hypercapnia) as the two factors. In all panels, the main effects were significant ($p_{genotype} < 0.001$ and $p_{exposure} < 0.001$ in all cases except panel C, $p_{exposure} = 0.033$) but the interaction was not ($p_{gen\ x\ exp} = 0.465$, 0.243 and 0.218 for panels A, B and C, respectively). Grouped bars that share a letter are not significantly different from one another, and the effect of exposure is indicated in the symbol legend. Values are means + SEM, $N = 10$.

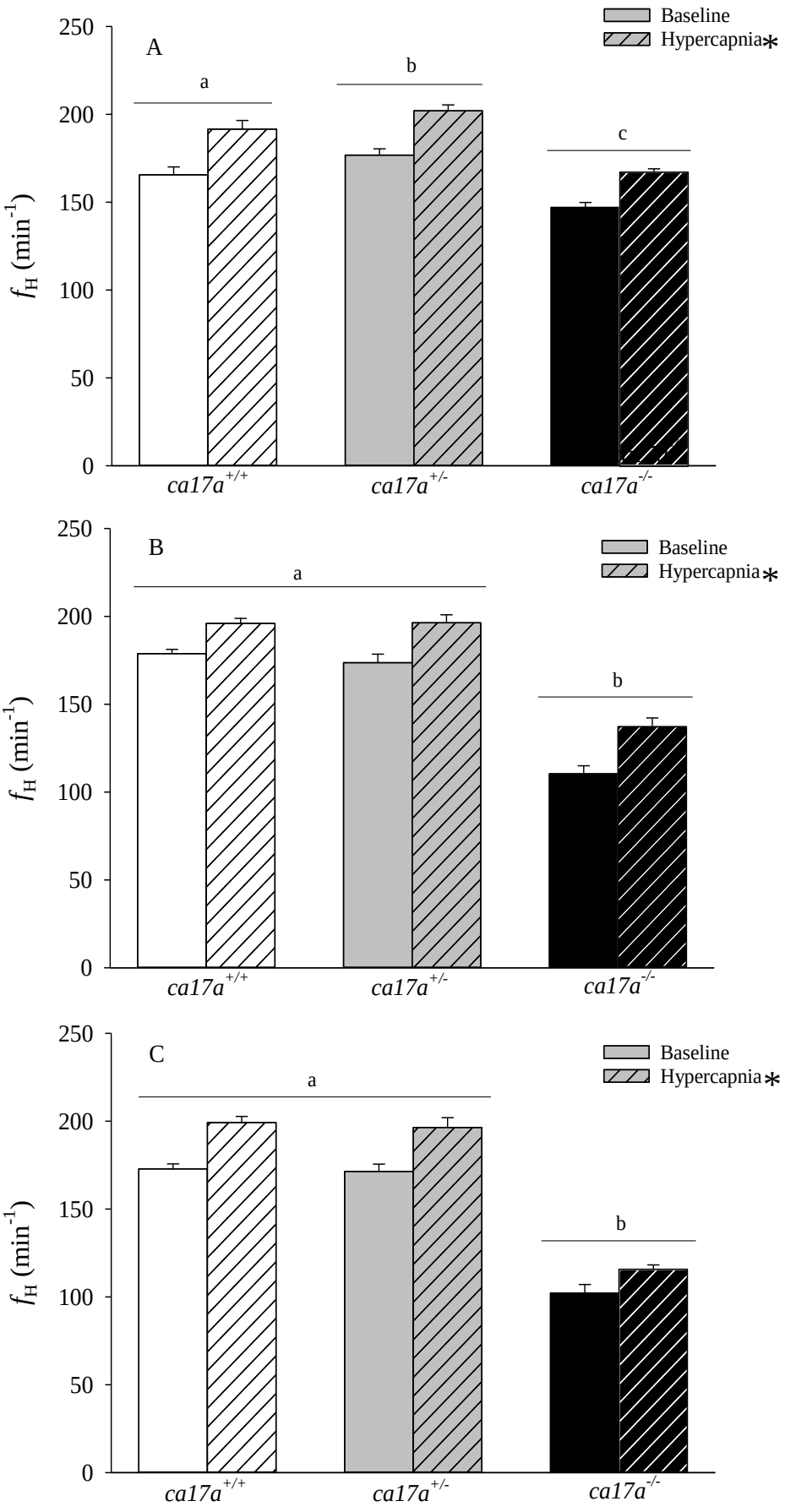

A
B
C
Baseline
Hypercapnia*
a
b
c
$f_H$ (min$^{-1}$)
250
200
150
100
50
0
ca17a$^{+/+}$
ca17a$^{+/-}$
ca17a$^{-/-}$

Figure 8. The effect of Ca17a knockout on (A) resting heart rate ($f_H$) in larval zebrafish (*Danio rerio*), and (B) duration of inactivity and (C) total distance moved by 8 dpf larvae under dark conditions. In panel A, a significant interaction was detected between genotype and age (two-way ANOVA, $p < 0.001$). Bars that share a letter are not significantly different from one another (lower case letters for wild-type fish and uppercase letters for mutants), and an asterisk indicates a significant effect of genotype at a given age. In panels B and C, no significant effect of genotype was detected (ANOVA, $p = 0.076$, 0.088 for panels B and C, respectively). Values are means + SEM, $N$ = 6-10 for panel A, and 13-15 for panels B and C.

.

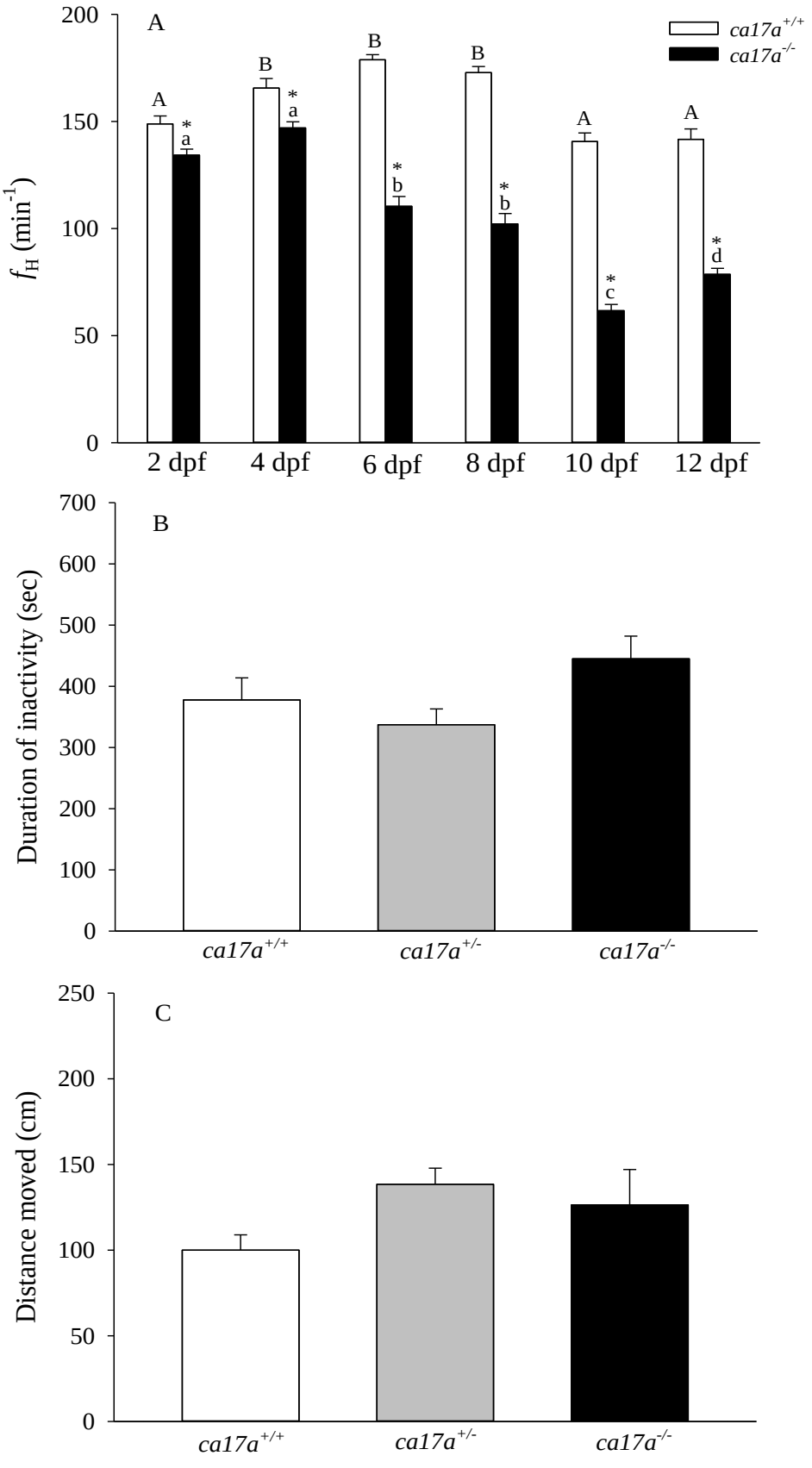
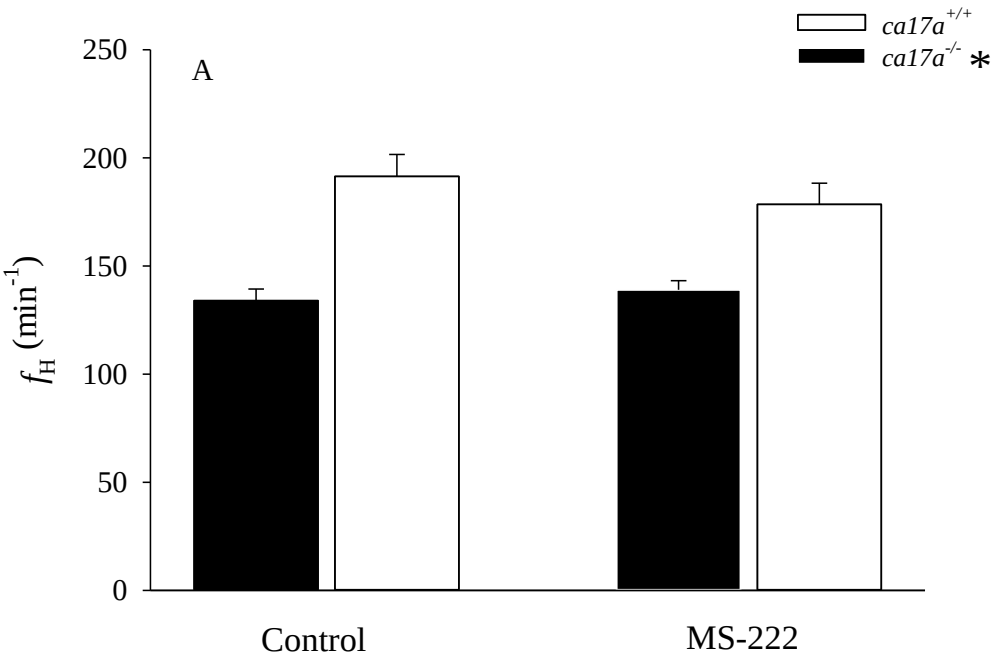

A
ca17a+/+
ca17a-/-
fH (min-1)
200
150
100
50
0
A
B
B
B
A
A
*a
*a
*b
*b
*c
*d
2 dpf
4 dpf
6 dpf
8 dpf
10 dpf
12 dpf
B
Duration of inactivity (sec)
700
600
500
400
300
200
100
0
ca17a+/+
ca17a+/-
ca17a-/-
C
Distance moved (cm)
250
200
150
100
50
0
ca17a+/+
ca17a+/-
ca17a-/-

Figure 9. The effects of (A) TRIS-buffered MS-222 (0.05 mg $mL^{-1}$), and (B) atropine ($10^{-4}$ M) and propranolol ($10^{-4}$ M) on heart rate ($f_H$) of *ca17a*$^{+/+}$ and *ca17a*$^{-/-}$ zebrafish (*Danio rerio*) larvae at 8 dpf. In panel A, a significant effect of genotype was identified (two-way ANOVA on log-transformed data, $p_{genotype} < 0.001$), but not of treatment nor the interaction of these factors ($p_{MS222} = 0.708$, $p_{gen\ x\ MS\text{-}222} = 0.219$). In panel B, both genotype and treatment had significant effects, but the interaction of these factors was not significant (two-way ANOVA on log-transformed data, $p_{genotype} < 0.001$, $p_{treatment} < 0.001$, $p_{gen\ x\ treat} = 0.820$). Grouped bars that share a letter are not significantly different from one another, and an asterisk in the legend indicates a significant effect of genotype within a treatment. Values are means + SEM, $N$ = 7-8.


A
ca17a+/+
ca17a-/- *
250
200
150
100
50
0
fH (min-1)
Control
MS-222

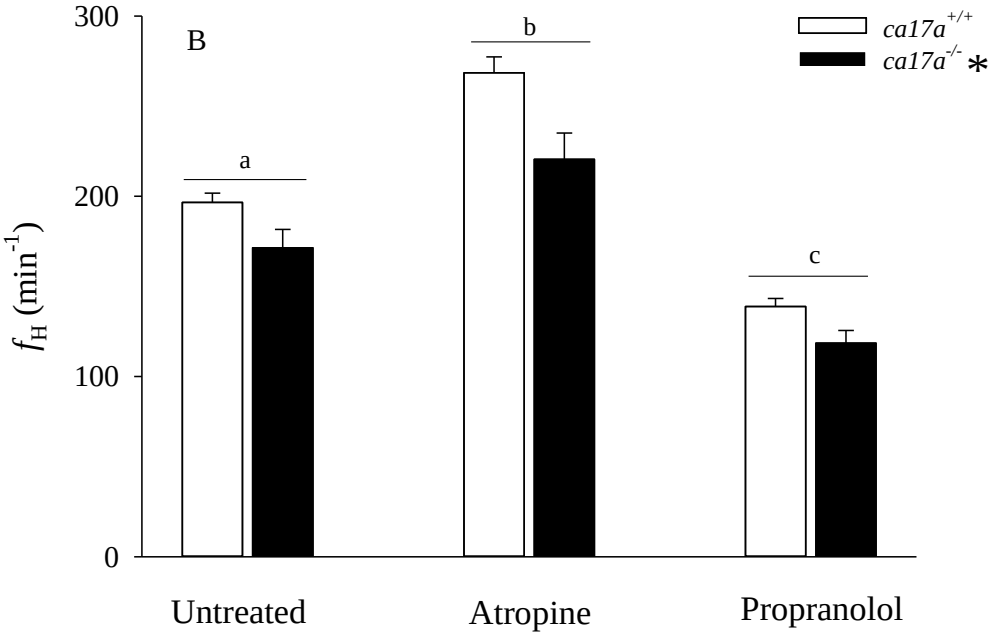
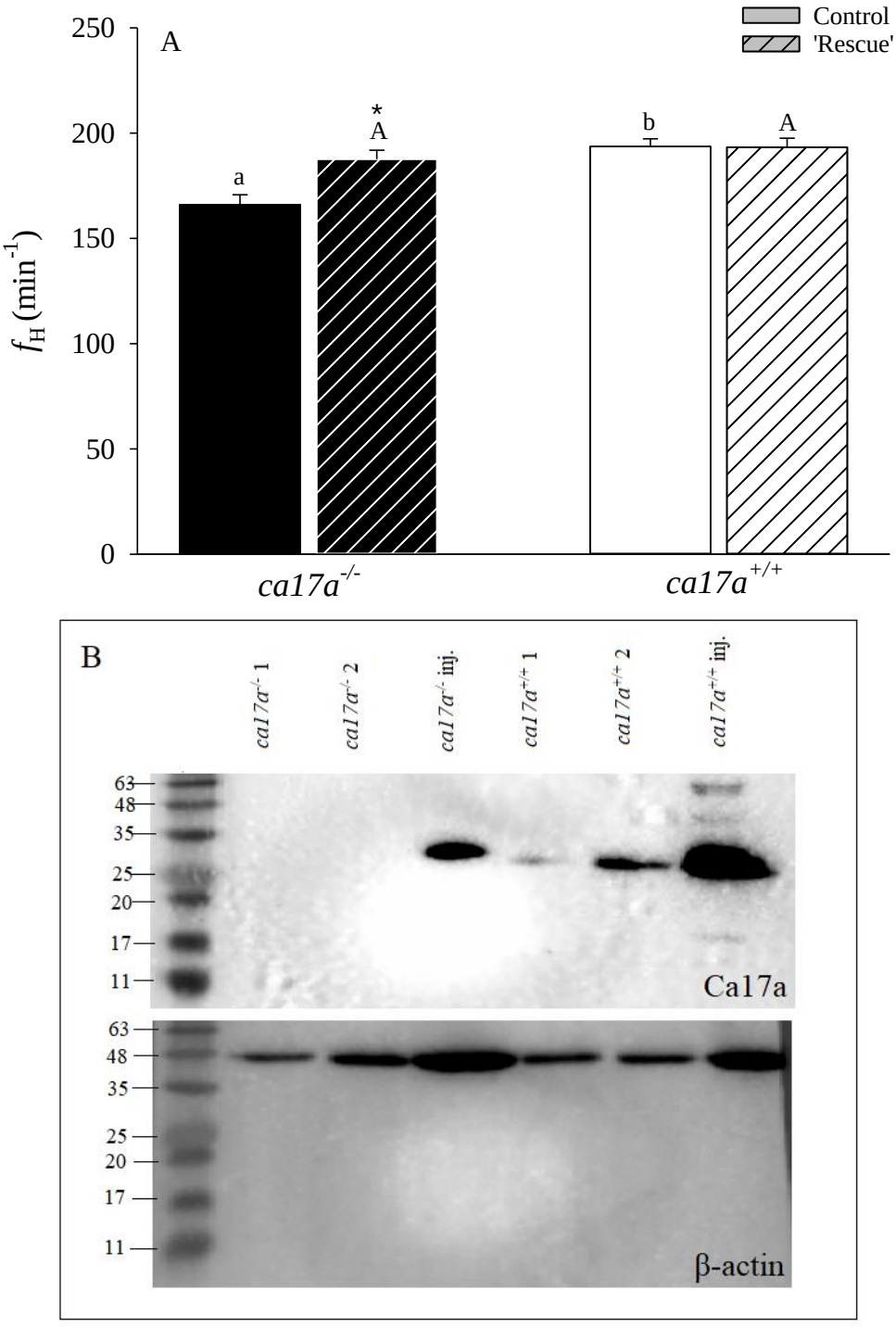

B
ca17a+/+
ca17a-/- *
300
200
100
0
fH (min-1)
a
b
c
Untreated
Atropine
Propranolol

Figure 10. Panel A presents the effect of genotype and *ca17a* mRNA 'rescue' on heart rate ($f_H$) in 4 dpf larval zebrafish (*Danio rerio*). Data were analyzed by two-way ANOVA with genotype and 'rescue' as the two factors, and a significant interaction between these factors was detected ($p = 0.013$). Bars that share a letter are not significantly different from one another (lower case letters for control, upper case letters for 'rescue') and an asterisk indicates a significant effect of 'rescue' within a genotype. Values are means + SEM, $N = 12$. Panel B presents western blots probed for Ca17a (upper blot) and ß-actin as a loading control (lower blot). Whereas *ca17a*$^{-/-}$ samples (1 and 2) lacked Ca17a protein expression, the sample from 'rescued' *ca17a*$^{-/-}$ larvae (*ca17a*$^{-/-}$ inj.) expressed Ca17a. Samples labelled *ca17a*$^{+/+}$ 1 and *ca17a*$^{-/-}$ 1 were for 9 dpf fish, and all remaining samples were for 4 dpf fish.


A
Control
'Rescue'
250
200
150
100
50
0
$f_H$ (min$^{-1}$)
a
*
A
b
A
ca17a$^{-/-}$
ca17a$^{+/+}$
B
ca17a$^{-/-}$ 1
ca17a$^{-/-}$ 2
ca17a$^{-/-}$ inj.
ca17a$^{+/+}$ 1
ca17a$^{+/+}$ 2
ca17a$^{+/+}$ inj.
63
48
35
25
20
17
11
Ca17a
β-actin

# 4 Discussion

## 4.1. *Overview*

In mammals, the Type I (glomus) cells of the carotid body are peripheral chemoreceptors of $O_2$, $CO_2$, and pH (reviewed by Gonzalez et al., 1994) that express CA (Nurse, 1990; Rigual et al., 1985). Inhibition of CA *in vivo* and *in vitro* reduces chemosensory responses (Black et al., 1971; Iturriaga et al., 1991; reviewed by Iturriaga, 1993). Previous studies on fish have implicated CA as a possible contributor to $CO_2$ chemoreception, owing to its role in catalyzing the hydration of $CO_2$ to $H^+$ and $HCO_3^-$ and its apparent expression in NECs of larval and adult zebrafish (Miller et al., 2014; Qin et al., 2010). Previous studies, using gene knockdown or pharmacological inhibition to prevent normal Ca17a function, found that Ca17a promotes the cardiac response (tachycardia) of larval zebrafish to hypercapnia (Miller et al., 2014). Miller et al. (2014) reported that morpholino knockdown was effective only up to 5 dpf. Acetazolamide was used successfully to inhibit Ca17a activity (Abdallah et al., 2015; Miller et al., 2014; Qin et al., 2010), but it inhibits all isoforms of CA making it impossible to distinguish isoform-specific effects.

To date, only a single study has examined the effects of Ca17a on the cardiac responses of larval zebrafish during hypercapnia, and no studies have investigated the ventilatory response. The purpose of this study was to use a line of Ca17a knockout zebrafish to investigate the role of this enzyme in the cardiorespiratory responses of zebrafish larvae to hypercapnia. The results showed that Ca17a indeed plays a role in the ventilatory responses to hypercapnia, with *ca17a*$^{-/-}$ fish showing an attenuated ventilatory response. However, the effects were complex and age-dependent differences existed. In contrast to the results of Miller et al. (2014) using gene knockdown, there was no effect of Ca17a knockout on the cardiac response to hypercapnia.

Interestingly, however, the baseline (resting) $f_H$ of the *ca17a*$^{-/-}$ fish was lower than that of *ca17a*$^{+/+}$ and *ca17a*$^{+/-}$ fish. Upon further investigation, $f_H$ was found to decrease as age increased, potentially explaining the early death of the *ca17a*$^{-/-}$ fish at ~14 dpf (Zimmer et al., 2020).

It should be noted that the effects of Ca17a knockout on cardiorespiratory responses evaluated in the present study do not necessarily provide direct evidence that Ca17a is involved in cellular (i.e., NEC) $CO_2$ chemoreception. Measuring $f_V$ and $f_H$ was merely a method to investigate downstream responses that arose from $CO_2$ chemoreception. While it is probable that any attenuation of these responses to hypercapnia is linked to loss of Ca17a function in the $CO_2$ chemoreceptors, there may also be influence from a number of other pathways linking $CO_2$ chemoreception and cardiorespiratory reflexes.

4.2 *The ventilatory response*

In the current study, larvae responded to the different levels of $CO_2$ with varying degrees of increased $f_V$. Ultimately, 1% $CO_2$ was chosen as the level of hypercapnia to be used in all subsequent experiments. In response to hypercapnia, all genotypes exhibited hyperventilation, classified in the present study as an increase in $f_V$. Although adult zebrafish exclusively increase ventilation amplitude in response to hypercapnia (Vulesevic et al., 2006), measurement of this variable is technically challenging in zebrafish larvae. Thus, in the absence of a technique to monitor amplitude, the present experiments relied solely on measurements of $f_V$, which was known to increase in response to hypercapnia (Koudrina et al., 2020).

Although response thresholds may vary, the hyperventilatory response to hypercapnia appears to be a universal trait in adult fishes (reviewed by (Gilmour, 2001). The hypercapnic ventilatory response is presumed to be ancestral because it is exhibited in the basally positioned hagfish (*Eptatretus stoutii*; Perry et al., 2009b). The ventilatory response to hypercapnia of larval

fish has been restricted to a single species, zebrafish (Koudrina et al., 2020). In that study, it was reported that larvae at 4 dpf exposed to 1.5% $CO_2$ experienced an increase in $f_V$ of approximately 30 breaths $min^{-1}$ (Koudrina et al., 2020), in rough agreement with the results of the current study using a slightly milder hypercapnic exposure. Notably, however, the baseline $f_V$ in the current study (~2 breaths $min^{-1}$) was considerably lower than the resting $f_V$ reported previously (10 – 30 breaths $min^{-1}$). As the fish aged, their ventilatory response to hypercapnia increased. This age dependent response also was observed in larval zebrafish exposed to hypoxia (Pan et al., 2019). The increase in $f_V$ in zebrafish exposed to hypercapnia appears to be unique to larvae; the $f_V$ of adult zebrafish is unaffected by 1% $CO_2$ (7.5 mm Hg; Porteus et al., 2020). Larval zebrafish appear to be less sensitive to $CO_2$ than adults with higher levels of $CO_2$ required to elicit hyperventilation as adults respond to $CO_2$ as high as 3.5 mm Hg (Vulesevic et al., 2006), whereas larval zebrafish only responded to 11.3 mm Hg (1.5%; Figs. 1 and 2).

It is debatable whether hyperventilation at an early developmental age is of physiological benefit because larvae younger than 14 dpf have an under-developed gill and rely predominantly on cutaneous gas exchange (Jonz and Nurse, 2005; Rombough, 2002). However, in a study on the hyperventilatory response of zebrafish larvae to hypoxia, $O_2$ was found to be extracted from the buccal cavity as early as 4 dpf (Pan et al., 2019). Although there are no similar studies on the hyperventilatory response of larvae to hypercapnia, it is known that they are able to excrete $CO_2$ as early as 3 hpf (Gilmour et al., 2009) and thus by analogy to $O_2$ uptake (Pan et al., 2019), the buccal cavity also may be a site of $CO_2$ excretion during early development.

At 8 dpf, the $f_V$ response to hypercapnia of the *ca17a*$^{-/-}$ fish was blunted. Thus, 8 dpf would appear to be the age where fish become dependent on Ca17a for $CO_2$ sensing or in promoting downstream responses associated with $CO_2$ sensing. At 6 dpf, the $f_V$ during

hypercapnia of *ca17a*$^{-/-}$ was not significantly different from that of either *ca17a*$^{+/+}$ or *ca17a*$^{+/-}$ fish (Fig. 4). It is unclear why *ca17a*$^{-/-}$ fish exhibited a significantly higher peak $f_V$ at 4 dpf than wild type or heterozygous larvae, an unexpected response that was maintained for at least 5 min of the hypercapnic exposure (Fig. 3). Ca17a catalyzes the hydration of $CO_2$ to $H^+$ and $HCO_3^-$. It has been hypothesized that TASK-2 $K^+$ channels are involved in the response to $CO_2$ (Koudrina et al., 2020; Niemeyer et al., 2010; Peña-Münzenmayer et al., 2014). Recently, Koudrina et al. (2020) demonstrated that fish lacking expression of one or both isoforms of TASK-2 exhibited attenuated $f_V$ responses to hypercapnia. Because TASK-2 is deactivated by low pH, the accumulation of $H^+$ from the catalyzed hydration of $CO_2$ would result in their deactivation and subsequent loss or delay of signal (Niemeyer et al., 2010; Peña-Münzenmayer et al., 2014). Notable, lack of Ca17a does not cease the hydration of $CO_2$ but rather, slows the process considerably (Abdallah et al., 2015). This factor may explain how *ca17a*$^{-/-}$ fish are still able to respond to hypercapnia.

Previous *in vitro* studies have demonstrated that NECs are $CO_2$ chemoreceptors (Abdallah et al., 2015; Qin et al., 2010) and there is indirect evidence linking them to the *in vivo* cardiorespiratory responses associated with hypercapnia (Koudrina et al., 2020; Miller et al., 2014). Thus, the present thesis tested the possibility that the blunting of the hypercapnic ventilatory response in CA17a knockouts reflected a decrease in the numbers of cutaneous NECs. However, the density of NECs was similar across genotypes and in keeping with a previous study (Coccimiglio and Jonz, 2012), decreased by approximately 50% from 4 to 8 dpf. As the larvae transition to branchial respiration, the cutaneous NECs are gradually lost while their numbers increase on the gill. Because *ca17a*$^{-/-}$ fish die around 14 dpf (Zimmer et al., 2020), when the larvae are more dependent on branchial respiration (Jonz and Nurse, 2005; Rombough,

2002), it was not possible to investigate the role of Ca17a in fish relying mostly on branchial gas transfer.

This study was the first to report the effects of hypercapnia on $f_V$ in larval zebrafish as a function of exposure time. A similar trend occurred in all genotypes, with a transient increase of $f_V$ lasting the first few minutes of hypercapnia, followed by a gradual decline, in some cases returning to a baseline frequency (Figs. 1 and 3). This transient response in fish resembles the response seen in rats (Lai et al., 1981) and humans (Schaefer et al., 1963) exposed to sustained hypercapnia. Dogs also show decreased $f_V$ during sustained hypercapnia, although a secondary rise was reported during the first week of exposure (Jennings and Chen, 1976). This reported acclimation may reflect a redistribution of $HCO_3^-$ out of cerebrospinal fluid and increased renal excretion of $HCO_3^-$ leading to a reduction of cerebral $[H^+]$, thereby reducing stimulation of chemoreceptors (Lumb and Horner, 2013). In fish, only one study has reported a transient decrease in $f_V$, during acute hypoxic exposure in larval zebrafish (Pan et al., 2019). Two possibilities for the transient responses to hypercapnia in larvae are that the NECs of larval fish have not yet developed a capacity to sustain a hyperventilatory response, or that larvae have not yet developed receptors, that may be present in older fish, that allow for a sustained hyperventilation during hypercapnia. Abdallah et al. (2015) examined the effect of $CO_2/H^+$ on the intracellular concentration of $Ca^{2+}$ and found that during both acidic hypercapnia and isocapnic acidosis there was a transient increase in $Ca^{2+}$ but that the response recovered well before the end of the stimulus period. Because the rise in $Ca^{2+}$ is believed to be linked to neurosecretion (Buckler and Vaughan-Jones, 1994), the transient rise in intracellular $Ca^{2+}$ may contribute to the decrease in $f_V$ over exposure time. A possible outcome of the decline in $f_V$ in fish may be reduced energy expenditure, because ventilating is energetically costly (Jones and

Schwarzfeld, 1974; Steffensen, 1985), at least in adult fish; the relative cost of ventilation in larvae is unknown.

Abdallah et al. (2015) reported that NEC $Ca^{2+}$ levels were unchanged during exposure to isohydric hypercapnia, data suggesting that NECs responded to extracellular $H^+$ rather than intracellular $H^+$ or $CO_2$. In contrast, Qin et al. (2010) observed membrane depolarization in NECs exposed to isohydric hypercapnia, indicating that NECs do indeed respond to $CO_2$. When cells were treated with ACTZ to block CA, this depolarization was blunted, meaning that the response was mediated by $pH_i$ rather than $pH_o$ (Qin et al., 2010). The discrepancy between these two studies may indicate that multiple types of $K^+$ channels are involved, with some channels being sensitive to $pH_i$, and others sensitive to $pH_o$ (reviewed by Perry and Tzaneva, 2016). The results also demonstrate that membrane depolarization may not always be accompanied by an increase in cytosolic $[Ca^{2+}]$. The present study did not compare these three conditions and therefore it is not known whether $CO_2$ or $H^+$ was responsible for eliciting the $f_V$ response, although it has generally been accepted that cardioventilatory responses reflect changes in external $CO_2$ (Miller et al., 2014; Perry and McKendry, 2001; Reid et al., 2000; Sundin et al., 2000).

4.3 *The cardiac response*

In the present study, all genotypes responded to hypercapnia with an increase in $f_H$, i.e., tachycardia. The degree to which $f_H$ increased was similar across genotypes, i.e., regardless of the presence or absence of Ca17a, thereby indicating that Ca17a is not required to elicit hypercapnic tachycardia, a result that contrasts with the findings of a previous study that used Ca17a knockdown (Miller et al., 2014). The different findings of the two studies may reflect compensatory responses to gene knockout that may not occur during gene knockdown (Zimmer

et al., 2019). An unexpected outcome of the current experiments was the observation that the baseline $f_H$ in *ca17a*$^{-/-}$ fish was lower than that of *ca17a*$^{+/+}$ and *ca17a*$^{+/-}$ fish. This phenomenon was further investigated, revealing that $f_H$ was significantly lower in knockout larvae at all ages to 12 dpf. Unfortunately, no fish survived to be tested at 14 dpf, and perhaps their early death can be associated with a cardiac output too low to support gas exchange and other vital functions, as premature death has also been observed in larval zebrafish lacking convection (Sehnert et al., 2002). To ask whether the lower baseline $f_H$ of *ca17a*$^{-/-}$ fish reflected a difference in intrinsic $f_H$ or a difference in autonomic regulation of $f_H$, $f_H$ was assessed in the presence of inhibitors of autonomic receptors. Atropine and propranolol were used to probe the possible influence of adrenergic and cholinergic tone, respectively, on the resting $f_H$ response in *ca17a*$^{-/-}$ fish. Atropine resulted in an increase in $f_H$ in both *ca17a*$^{+/+}$ and *ca17a*$^{-/-}$ fish, with the difference in $f_H$ remaining between genotypes. Atropine is a muscarinic cholinergic receptor blocker, and the persistence of the genotype disparity following atropine treatment indicated that the difference was not due to cholinergic tone. Similarly, propranolol decreased $f_H$ for both genotypes, but the difference between genotypes persisted. Propranolol is a non-specific β-adrenergic agonist. In the absence of a genotype-specific effect on $f_H$, it is likely that differences in adrenergic tone were not responsible for the reduced resting $f_H$ of knockout fish.

Thus, the knockout of Ca17a appears to affect intrinsic heart rate or non-autonomic cardiac regulators. Other studies have shown that anesthetic may affect $f_H$ (Mann et al., 2010), although in the present study, $f_H$ of *ca17a*$^{+/+}$ and *ca17a*$^{-/-}$ fish exposed to MS-222 did not differ from control values. To assess whether the lower $f_H$ of knockout fish could be attributed specifically to the absence of Ca17a, a final experiment involved injecting *ca17a*$^{+/+}$ and *ca17a*$^{-/-}$ fish with *ca17a* mRNA in an attempt to 'rescue' $f_H$. The *ca17a*$^{-/-}$ fish injected with *ca17a* mRNA

exhibited normal (wild type) resting $f_H$, i.e., the wild-type cardiac phenotype was rescued by *ca17a* mRNA injection. These results demonstrate that the lowered $f_H$ was indeed a specific effect of Ca17a knockout and that off-target mutations associated with generation of the mutant line were unlikely to be contributing to the reduced $f_H$ of *ca17a*$^{-/-}$ fish. However, additional research is needed to understand the mechanistic basis of Ca17a knockout effects on resting $f_H$.

A single previous study examined the effect of hypercapnia and Ca17a on cardiac responses in zebrafish (Miller et al., 2014). In that study, an increase in $f_H$ in response to hypercapnia also was observed although unlike in this study, there was a lack of hypercapnic tachycardia after inhibition of Ca17a or morpholino knockdown (Miller et al., 2014); indeed, fish with diminished Ca17a function experienced hypercapnic bradycardia. The differences between the two studies are puzzling although it is likely that methodological differences were an important contributor. Indeed, the various techniques used to reduce CA function (pharmacological inhibition, knockdown and knockout) all have limitations. Acetazolamide inhibits all isoforms of CA, and while Ca17a has been identified as the 'workhorse' CA isoform, with the highest turnover rate (reviewed by Lindskog, 1997), it is possible that other isoforms such as Ca17b, which catalyzes the dehydration of $HCO_3^-$ in blood into diffusible $CO_2$, may be involved. In fact, Miller (2013) performed knockdown of Ca17b as well as Ca17a and found that both resulted in attenuated $f_H$ responses to hypercapnia. Although the goal of knockdown is for complete LOF of a protein, LOF often will be partial (reviewed by Zimmer et al., 2019). Additionally, gene knockdown is known to produce off-target effects arising from unintended genes being targeted or causing toxicity resulting in non-specific effects (reviewed by Zimmer et al., 2019). Thus, while knockdown may be preferable to inhibition with ACTZ, it has its own limitation. Knockout also does not come without limitations. For example, there is the possibility

of off-target mutations, although these may be 2-fold lower than on-target mutations so long as there is one or more bp mismatch compared to the on-target site (reviewed by Zimmer et al., 2019). Off-target mutations can be segregated through breeding during the generation of a homozygous line (reviewed by Zimmer et al., 2019), however the present study required breeding from heterozygous parents. Breeding using heterozygous parents can be problematic if they are able to pass on sufficient maternal wild type mRNA of the target gene to the mutant offspring (reviewed by Zimmer et al., 2019). Western blots (Fig 10B) and IHC (results not shown) confirmed that Ca17a was not present in *ca17a*$^{-/-}$ fish bred from heterozygous parents. Additionally, changes in the genome resulting from knockout can lead to transcriptional adaptation, potentially causing upregulation of another gene resulting in compensatory gene regulation (reviewed by Zimmer et al., 2019).

Several studies that have investigated the effect of hypoxia on $f_H$ in larval zebrafish with the responses being highly variable depending on factors such as age, environmental conditions, level of hypoxia or the duration of exposure (discussed in Hughes and Perry, in press). In some cases the effect was a bradycardic response (Barrionuevo and Burggren, 1999; Steele et al., 2009; Steele et al., 2011a), while others showed tachycardia (Jacob et al., 2002; Koudrina et al., 2020). In the present study, as in previous experiments (Koudrina et al., 2020; Miller et al., 2014), the cardiac response to hypercapnia was tachycardia. It should be noted that there are alternative methods of assessing cardiac function, such as by measuring stroke volume or cardiac output (Kopp et al., 2006; Steele et al., 2011b), which clearly are of importance. Owing to the complexity of measuring stroke volume and cardiac output, such analyses were beyond the scope of this project and therefore $f_H$ was the sole downstream cardiac response investigated. There is currently no concrete explanation as to why larval zebrafish respond to hypercapnia via

tachycardia. A study by Gilmour et al. (2009) used knockdown of Ca17b to show that $CO_2$ excretion was diminished when Ca17b activity was reduced. Owing to the specific location of Ca17b in RBCs, it was suggested that larval zebrafish were likely utilizing RBCs in $CO_2$ excretion (Gilmour et al., 2009). Because branchial respiration does not predominate until ~14 dpf (Jonz and Nurse, 2005; Rombough, 2002), $CO_2$ excretion likely was occurring over the whole-body and/or the yolk sac surfaces (Gilmour et al., 2009). Furthermore, larval zebrafish at 4 dpf rely on internal convection (blood flow) to assist gas transfer (Hughes et al., 2019). By preventing internal convection using knockdown of vascular endothelial growth factor, it was found that $CO_2$ excretion rates were diminished (Hughes et al., 2019). It was concluded that circulation can aid in cutaneous gas exchange by sustaining diffusion gradients as $CO_2$ is moved away from areas of accumulation and areas that are unreachable by diffusion alone. Because circulating RBCs appear to be important for normal rates of $CO_2$ excretion even at early stages of development (Gilmour et al., 2009), internal convection promoted by tachycardia could benefit $CO_2$ excretion.

### 4.4 *Concluding remarks and future directions*

The present study demonstrated that Ca17a is involved in the ventilatory responses to hypercapnia in zebrafish larvae at 8 dpf but is not required for the cardiac responses to elevated $CO_2$. However, it appears that Ca17a plays a role in setting the resting $f_H$ of larval zebrafish, which was an unexpected result.

Probing the cardiorespiratory responses to hypercapnia in adult fish lacking Ca17a was not possible in this study because knockout fish perished during larval development. Future studies should investigate responses in adults, should a knockout line that survives to maturity be

generated. Indeed, it would be informative to collect $f_H$ measurements of adult wild type zebrafish exposed to hypercapnia as data have yet to be published.

The unexpected effect of loss of Ca17a function on baseline $f_H$ raises questions concerning the distribution of Ca17a in the heart. Although *ca17a* mRNA has been reported to be present in the heart of adult zebrafish (Lin et al., 2008), its distribution within the heart itself has yet to be explored. The use of IHC staining to determine whether Ca17a is expressed in pacemaker cells, for example, would be useful in investigating the mechanisms underlying the effects of Ca17a knockout on $f_H$.

A broader question concerns the specific stimulus for cardiorespiratory responses to hypercapnia. Because currently there is no conclusive evidence to indicate whether $CO_2$ or $H^+$ is responsible for the chemosensory response to hypercapnia, evaluation of $f_V$ and $f_H$ in response to isohydric hypercapnia, isocapnic acidosis, and acidic hypercapnia, as in studies on isolated NECs (Abdallah et al., 2015; Qin et al., 2010), would be useful in determining the specific stimulus.

In conclusion, the present study provided important groundwork for future studies focused on the role of Ca17a in cardiorespiratory responses to hypercapnia. Arguably the most intriguing result of the present study was the effect of Ca17a knockout on resting $f_H$, a result that implies a specific role for Ca17a in setting heart rate and warrants further study.

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