Calcium carbonate production by teleost fish: an investigation into the effects of temperature and dietary calcium intake

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Abstract

The marine environment is hyperosmotic to the tissues of marine teleost fish, which results in passive water loss and ion gain throughout their lifetime. In order to avoid dehydration, marine teleosts drink seawater. Ingested seawater travels through the digestive tract, where it is manipulated in order to absorb water and selected ions. Calcium carbonate ($CaCO_3$) is precipitated as part of this process, and aids water absorption by removing Ca^{2+} (calcium) and HCO_3^- (bicarbonate) ions from solution. This precipitate (hereby referred to as carbonate), which also contains some $MgCO_3$, is then excreted to the environment as a waste product, where it can dissolve or become part of the sediment. The precipitation and dissolution of carbonate forms the marine inorganic carbon cycle, which we have only recently understood that fish form a significant part. In chapter one of this thesis, I have reviewed the literature to explore the many factors that affect the production of gut carbonates by fish, including absolute quantity, composition and how they affect the fate of carbonate after excretion. In chapter two I have experimentally investigated the effect of low temperature, from 3 to 14 °C, on the quantity and composition of carbonate excreted by lumpfish, *Cyclopterus lumpus*. Between 3 and 7 °C, carbonate excretion increased with a Q_{10} of 3.50, which is comparable to other species, and had no effect on the incorporation of $MgCO_3$. This confirms that carbonate excretion rate responds to temperatures <10 °C in a similar way to the higher temperatures used in other studies. The consistent carbonate composition across tested temperatures suggests that any changes in dissolution rate of carbonates is likely to be due to temperature-associated changes in Ω_{CaCO_3}. In chapter three I investigated the effect of diets supplemented with calcium salts ($CaCO_3$, $Ca_3(PO_4)_2$, and $CaCl_2$), two of which had elevated dietary pH buffering capacity, on the digestive physiology and carbonate production in freshwater-acclimated rainbow trout, *Oncorhynchus mykiss*. We found that consumption of any diet by these freshwater fish resulted in the alkalinisation of intestinal fluid with high levels of HCO_3^- ions resulting in the production and excretion of precipitated calcium carbonate minerals, previously thought to be exclusive to marine teleosts, which was particularly high in fish fed the $CaCl_2$ or $CaCO_3$ diets. This is the first known report of

intestinal carbonate excretion by a freshwater fish. These results have consequences for modelling of global carbonate production by fish and potentially the production of aquaculture feeds.

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Chapter 1: Literature review: The factors influencing and environmental consequences of calcium carbonate production by fish

Introduction to ion regulation in teleosts

Osmoregulating and osmoconforming are two strategies used by marine animals to cope with living in seawater (~33-35 ppt/ ~1050mOsm/l). Hagfishes, elasmobranchs, and most marine invertebrates are osmoconformers, with hagfishes and many invertebrates having bodily sodium chloride (NaCl) concentrations similar to that of seawater. All other vertebrates have bodily NaCl concentrations ~30% that of seawater, with elasmobranchs supporting slightly hyperosmotic tissues by maintaining highly elevated levels of the compatible organic osmolytes urea and methylamines (Yancey *et al.*, 1982; Evans, Piermarini and Choe, 2005; Hwang and Lin, 2013).

As osmoregulators, marine teleost fish maintain tissue osmolality at around a third that of seawater (Shehadeh and Gordon, 1969; Evans, Piermarini and Choe, 2005). Their ion- and osmoregulatory processes can be characterised as salt secretion and water retention (Kültz, 2015). Much ion exchange occurs at the gills, which have the advantage of large surface area and much higher flow rates compared to urinary flow rate in the kidney, therefore removing the issue of accumulated excreted products affecting osmotic gradients for excretion (Evans, Piermarini and Choe, 2005). During development, the gills are used for ion regulation before they are used for oxygen uptake (Rombough, 2002; Fu *et al.*, 2010; Zimmer, Wright and Wood, 2014). It has therefore been suggested that ion regulation was the driving force for gill development, which were later co-opted for gas exchange (Baker *et al.*, 2015), as opposed to the traditional view that gills developed primarily for the latter (Gans, 1989; Rombough, 2007).

As teleosts are unable to absorb water directly from the environment, they must drink hyperosmotic seawater and extract fluid from this to avoid dehydration. Seawater travels through the digestive tract where it is manipulated to facilitate absorption of water and selected ions. Part of this process is the precipitation of calcium carbonate ($CaCO_3$), which reduces the osmolality of intestinal fluid. This process is affected by many exogenous and endogenous factors and can have quantitatively important impacts on the marine inorganic carbon cycle. The aim of this literature review is to

elucidate the factors that affect the production of $CaCO_3$ by fish, including the absolute quantity of $CaCO_3$, and its composition. Where appropriate, I will also cover how these factors affect the potential for the dissolution of $CaCO_3$ in the environment.

Homeostasis of main seawater ions

Chloride (Cl^-) and Sodium (Na^+) are the most abundant ions in seawater, with concentrations of ~546 and ~469 mM respectively (Pilson, 1998). Potassium (K^+) concentration is 9.3 mM in seawater (Hwang and Lin, 2013) and is intricately linked with NaCl excretion. Teleosts primarily excrete NaCl at the mitochondria-rich (MR) cells in the gill, using three main transporters. Na^+-K^+-ATPase (NKA) and Na^+-K^+-$2Cl^-$ cotransporter (NKCC) are located in the basolateral membrane, where NKA energetically transports 2 K^+ and 3 Na^+ into and out of the cell, respectively, while NKCC uses the resulting electrochemical gradient for Na^+ to facilitate cotransport of Na^+, K^+ and $2Cl^-$ into the cell. The apical membrane contains cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels, which facilitate accumulated intracellular Cl^- to leave the cell for the external medium down its trans-membrane electrochemical gradient. The movement of Cl^- through the apical membrane creates an electrochemical gradient across the whole epithelium which provides the driving force for Na^+ to move from the interstitial fluid to the environment through the paracellular pathway, specifically between MR cells and their neighbouring accessory cells, which is much more permeable to Na^+ ions than tight junctions across the rest of the epithelium. Recycling of K^+ across the basolateral membrane is achieved via the inward rectifier potassium channel (eKir) (Tse, Au and Wong, 2006), while excretion of excess K^+ is predominantly through renal outer medullary K^+ (ROMK) channels expressed at the gill (Furukawa *et al.*, 2012).

Magnesium (Mg^{2+}) and sulphate (SO_4^{2-}) ions are the second most abundant cation and anion in seawater, with concentrations of 53 and 28 mM respectively (Pilson, 1998; Marshall and Grosell, 2006; Kato *et al.*, 2009). Both are predominantly excreted via the kidney, at concentrations of ~74 mM SO_4^{2-} and ~95 mM Mg^{2+} in isosmotic urine (McDonald and Grosell, 2006). Pathways for SO_4^{2-} and Mg^{2+} excretion are not as comprehensively elucidated as those for Na^+ and Cl^-. Sulphate excretion appears to be predominantly mediated by apical Cl^- exchange via the transporter mfSlc26a6A, which decreases intracellular negative charge (Kato *et al.*, 2009). Maintenance of intracellular negative charge to allow apical SO_4^{2-} excretion is powered by basolateral

NKA, with supply of intracellular SO_4^{2-} facilitated by basolateral HCO_3^- exchange (Kato *et al.*, 2009). Excretion of Mg^{2+} is through exocytosis of Mg^{2+} rich vacuoles, within which Mg^{2+} accumulation is facilitated by a magnesium transporter, likely via Na^+ exchange (Islam *et al.*, 2013). Urinary SO_4^{2-} and Mg^{2+} concentration is further increased by Na^+ and Cl^- reabsorption in the distal tubule and urinary bladder, stimulating water uptake (solute-coupled water absorption), while maintaining urine isosmolality (Howe and Gutknecht, 1978; Gans, 1989; McDonald and Grosell, 2006).

Regulation of acid-base relevant ions

As well as regulating bodily concentrations of the inorganic ions in seawater, marine teleosts tightly regulate intra- and extra-cellular pH. Maintaining acid-base balance is important for processes including enzyme activity, as well as respiratory gas (Montgomery *et al.*, 2019) and ion transport (Evans, Piermarini and Choe, 2005). Four primary ions/ gases involved in acid-base balance are H^+, HCO_3^- and NH_3/NH_4^+. Ammonia gas (NH_3) and ammonium ion (NH_4^+) are hereby referred to as ammonia if referring to total ammonia (the sum of both), and by their chemical symbol if referring to one specifically. Sources of acid-base disturbance include, but are not limited to, exhaustive exercise (Wood, 1991) and gastric digestion, which causes the "alkaline tide" (Bucking and Wood, 2008; Cooper and Wilson, 2008). Due to ventilation's role of absorbing O_2 from a low O_2 medium, which causes low CO_2 and HCO_3^- in the blood, fish are unable to effectively adjust plasma pH by respiratory compensation, and instead must use metabolic compensation (regulation of H^+ and HCO_3^- excretion/ uptake rates) (Perry and Gilmour, 2006). The gills are normally the primary site of acid-base regulation, by contributing upwards of 90% of exchange of acid-base relevant ions to and from the environment (Claiborne, Edwards and Morrison-Shetlar, 2002).

Acidosis is a drop in blood pH, caused by an increase in the concentration of H^+ in the blood. This may occur when fish are exposed to high environmental CO_2 conditions, resulting in uptake of CO_2 into the blood and a drop in blood pH. Excess H^+ in the blood is therefore subsequently exchanged with Na^+ in seawater at the gill via the Na^+/H^+ exchanger (NHE) (Evans, Piermarini and Choe, 2005). Fish also utilise branchial accumulation of HCO_3^- to restore blood pH to normal within hours to days (Heuer and Grosell, 2014). Branchial HCO_3^- absorption appears to be via HCO_3^-/Cl^-

exchange or HCO_3^-/ Na^+ cotransport, through a variety of proteins in the SLC26 and SLC4 families (Heuer and Grosell, 2014).

Alkalosis is an increase in blood pH, caused by elevated concentrations of bases in the blood, such as HCO_3^- or NH_4^+. An alkalosis may also occur when a fish that has been exposed to high CO_2, and has compensated for this by accumulating HCO_3^-, and then returns to low CO_2 conditions, resulting in reduction of blood CO_2, while blood HCO_3^- temporarily remains high. As previously mentioned, fish are unable to utilise respiratory compensation, and thus are unable to retain CO_2 to compensate for alkalosis (Bucking and Wood, 2008; Cooper and Wilson, 2008). While the mechanisms for base excretion in marine teleosts are not as well understood as base uptake, it appears that marine fish are able to rapidly increase net base efflux following alkalosis (Lonthair, Dichiera and Esbaugh, 2020). The intestine may also play a role in recovering from alkalosis, as elevated blood HCO_3^- stimulates intestinal HCO_3^- secretion, reducing HCO_3^- concentration in the blood (Grosell *et al.*, 2005; Taylor *et al.*, 2007).

Amino acids are the monomeric subunit of proteins. Protein catabolism is the primary source of ammonia to teleosts, with protein sourced from muscle breakdown or dietary intake that is in excess of the requirements for protein synthesis and growth. While some of these amino acids are used for building tissues, amino acids are also used as a carbon source. In order to be used as a carbon source, amino acids are deaminated, producing carbon backbones (which are used as a precursor for carbohydrates) and ammonia (Evans, Piermarini and Choe, 2005). Ammonia is highly toxic (Wright and Wood, 2012), so it cannot be stored in the body (Larsen *et al.*, 2014) and must therefore be energetically converted to less toxic forms or excreted. Due to ammonia's high solubility in blood and seawater, as well as its generally strong concentration gradient to the environment, almost all teleosts are ammonotelic, meaning they excrete most of their nitrogenous waste as ammonia at no additional energetic cost.

While less is known about ammonia excretion in seawater compared to freshwater, similar principles are likely to apply, despite differences in external ion concentrations and gill ion permeability (Evans, 1984). Ammonia is excreted at the gill through rhesus (Rh) channel proteins, facilitated by Na^+ uptake. In freshwater, ammonia excretion at

the gill is enhanced by the acidification of the boundary layer by excreted metabolic CO_2 (Wright, Randall and Perry, 1989; Wilson *et al.*, 1994). Within this boundary layer next to the gills, excreted NH_3 reacts with H^+ under acidic conditions to form NH_4^+, restoring the strong concentration gradient for NH_3 moving out of the body. However, this effect may be reduced in seawater due to elevated alkalinity when compared to the majority of freshwater environments, limiting CO_2's acidifying effect through the buffering effect of alkalinity.

Ingested seawater

Due to the osmotic stress of living in a hyperosmotic environment (water loss and ion gain), marine fish are adapted to drink seawater, typically ~2-5 ml/kg/h (Grosell, 2007), in order to maintain internal osmolality. The osmolality of ingested seawater must be reduced to facilitate water absorption which can only occur against osmotic gradients of up to 35 mOsm/l (Genz, Esbaugh and Grosell, 2011). Desalinisation occurs in the oesophagus, where osmolality is reduced from ~1050 to ~500 mOsm/l (Grosell, 2019). Chloride absorption is thought to occur via apical bicarbonate (HCO_3^-) exchange, and basolateral HCO_3^- exchange and/ or a Cl^- channel. Sodium absorption is via apical H^+ exchange and basolateral K^+ exchange. Further Na^+ transport, through the paracellular pathway, is driven by the negative transepithelial potential to the blood generated by Cl^- transport (Esbaugh and Grosell, 2014).

Ingested seawater is acidified in the stomach to pH ~2-4, as this is the optimal pH for digestive enzymes (Johnston *et al.*, 2007). The reduction in gastric pH is achieved by secretion of hydrochloric acid (HCl) by oxyntopeptic cells that line the stomach, which must secrete an equimolar amount of HCO_3^- into the blood to balance intracellular pH. Due to the duration and magnitude of this alkalosis, gastric acid secretion by rainbow trout may cause the largest acid-base disturbance recorded in vertebrates (Urbina and Wilson, in preparation). The drop in pH strongly affects the equilibrium of the carbonate system in ingested seawater, converting all HCO_3^- and CO_3^{2-} to CO_2. Acidified seawater therefore enters the intestine with chyme (if the animal is feeding) at low pH and HCO_3^-, with an osmolality of ~500 mOsm/l (Grosell, 2019).

Fluid absorption in the intestine is facilitated by three processes: NaCl cotransport from the gut fluid, HCO_3^- secretion and Cl^- absorption via Cl^-/HCO_3^- exchange, and alkaline precipitation of $CaCO_3$ (Cooper, Whittamore and Wilson, 2010). In combination, these

three processes allow marine fish to absorb ~60-85% of ingested seawater (Grosell, 2007).

Salt absorption in the intestine is, in many ways, similar to salt excretion at the gills. Like at the gills, NKA and NKCC are involved in the transport of Na^+, Cl^- and K^+, while there is also involvement of a Na^+/Cl^- (NC) cotransport system. Basolateral NKA transports two K^+ into the cell in exchange for three Na^+, which leads to low concentrations of Na^+ and accumulation of negative charge within the intestinal cells (Grosell, 2007). This electrochemical Na^+ gradient leads to activation of the NKCC and NC cotransport systems, leading to absorption of Na^+, Cl^- and K^+ from the intestinal fluid, across the apical membrane (Grosell, 2007). Water is absorbed down the resulting osmotic gradient (solute-coupled water transport), and the salt absorbed by the intestine is then actively excreted at the gills (Larsen *et al.*, 2014).

Further Cl^- absorption from the intestinal fluid is facilitated by Cl^-/HCO_3^- exchange. The HCO_3^- used in this exchange is from two sources: basolateral absorption via Na^+/HCO_3^- (NBC) cotransport, and hydration of metabolic CO_2 by carbonic anhydrase (CA) within the cell itself (Tresguerres, Milsom and Perry, 2019). As the hydration of CO_2 produces H^+ as well as HCO_3^-, equimolar H^+ must be excreted from the cell to maintain intracellular acid-base balance. This excretion of H^+ is across the basolateral membrane, likely via NHE (Grosell and Genz, 2006; Grosell and Taylor, 2007; Gilmour *et al.*, 2012), followed by excretion of H^+ at the gills. The contribution of HCO_3^- of intracellular (hydration of metabolic CO_2 by CA) and extracellular (transport of HCO_3^- from the blood) source likely varies between species. In sea bass, *Dicentrarchus labrax*, ~75-80% of intestinal HCO_3^- secreted is of extracellular origin (Faggio *et al.*, 2011).

As a consequence of intestinal HCO_3^- secretion HCO_3^- accumulates in the intestinal lumen, up to a concentration of 100 mM (Wilson *et al.*, 1996). This high concentration of HCO_3^- creates an alkaline environment, with a pH of ~8.2-9.2 (Wilson *et al.*, 2009). This intestinal environment strongly favours precipitation of $CaCO_3$ and $MgCO_3$, with saturation state (Ω) of high magnesium calcite of ~10-360 (calculated in CO2Sys_v2.3 assuming a solubility 1.95 times that of aragonite, pH = 8.2-9.2, temperature = 15 °C, salinity = 11 ppt, TCO_2 = 30,000-150,000 µmol/kg, K1, K2 from Mehrbach *et al.*, 1973, KHSO$_4$ from Dickson, 1990, KHF from Perez and Fraga, 1987 – these constants are

used with CO2sys throughout)(Woosley, Millero and Grosell, 2012). Precipitation of $CaCO_3$ in the gut aids water absorption by reducing luminal osmolality by 70-100 mOsm/kg as a result of the removal of Ca^{2+} and HCO_3^- from solution (Wilson, Wilson and Grosell, 2002; Grosell *et al.*, 2009).

Secretion of H^+ into the intestinal lumen also occurs, equal to approximately 10% of net base secretion (Guffey, Esbaugh and Grosell, 2011; Grosell, 2019). While this seems counterintuitive, this likely serves to reduce intestinal HCO_3^- concentration (by dehydration of HCO_3^- to H_2O and CO_2) in distal portions of the gut where Ca^{2+} concentration may become limiting for calcification (Grosell *et al.*, 2009). This reduction in HCO_3^- concentration contributes to reduced osmolality, therefore allowing for more water absorption than in the absence of H^+ secretion. Following these transport processes and $CaCO_3$ precipitation, the remaining intestinal fluid and carbonates are excreted from the intestine.

As high rates of intestinal HCO_3^- secretion are characteristic of marine fish, the hormones associated with seawater acclimation in euryhaline fish are implicated in regulating intestinal HCO_3^- secretion (McCormick, 2001). These include cortisol and growth hormone (McCormick, 2001). Additionally, guanylin appears to play an intestine-specific role in intestinal water absorption and carbonate production by stimulating HCO_3^- secretion (Takei, Wong and Ando, 2019), while prolactin appears to inhibit HCO_3^- secretion (Ferlazzo *et al.*, 2012) . Parathyroid hormone-related protein (PTHrP) and stanniocalcin-1 (STC1) antagonistically control both Ca^{2+} and HCO_3^- in the intestine, with PTHrP reducing HCO_3^- secretion and increasing Ca^{2+} uptake, while STC1 promotes secretion of both Ca^{2+} and HCO_3^- (Fuentes, Power and Canário, 2010). Calcification in the intestine of marine fish is affected by a host of endo- and exogenous factors. Precipitate formation, and upon excretion its dissolution, also affect ocean chemistry. Elucidation of these is the primary goal of this review.

Carbonate products

The mucus-coated, carbonate-containing pellets excreted by starved fish vary in shape and size between individuals and species (Perry *et al.*, 2011). The mucus coating of these pellets is rapidly degraded in experimental procedures by sodium hypochlorite (NaClO), and is also thought to degrade rapidly in seawater (Wilson *et al.*, 2009; Perry *et al.*, 2011). Upon degradation of the mucus coating, carbonate

pellets up to 2 mm in size are released, which can be further disaggregated into crystallites and polycrystallite aggregates of varied size and morphology, mostly of less than 2 μm diameter (Perry *et al.*, 2011). These crystallites include a variety of morphologies, including ellipsoids, spheres, rhombohedra, dumbbells, straw-bundles and rods, among others, that are made up of Mg-calcites, aragonite and/ or amorphous carbonate phases (Perry *et al.*, 2011; Salter, Perry and Wilson, 2012; Foran, Weiner and Fine, 2013; Salter *et al.*, 2017). The carbonate morphology is consistent within species and family regardless of temperature (Salter, Perry and Smith, 2019), and bears a loose relationship with magnesium content (Salter *et al.*, 2018). Magnesium content of precipitates is important because it controls their solubility, and therefore the fate of carbonates post-excretion. Amorphous carbonates are particularly soluble (two orders of magnitude more so than calcite (Purgstaller *et al.*, 2019)) and therefore dissolve rapidly after excretion (Foran, Weiner and Fine, 2013).

<u>Environmental effects of carbonate precipitation and dissolution</u>

The oceans take up ~25% of anthropogenically produced CO_2 (Watson *et al.*, 2020), which reduces the accumulation of CO_2 in the atmosphere and therefore reduces the planetary greenhouse effect. Oceanic CO_2 uptake is facilitated by three processes: the solubility pump, the carbonate pump (the inorganic carbon cycle) and the biological pump (the organic carbon cycle). The process of calcification and the subsequent dissolution of $CaCO_3$ forms the inorganic carbon cycle. Calcification and dissolution of $CaCO_3$ in surface waters are intrinsically linked with oceanic CO_2 uptake due to these process' effects on the concentrations of aqueous CO_2, protons (H^+), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}). When dissolved in water, carbon dioxide (CO_2) is hydrated in the following reaction:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+$$
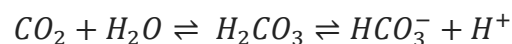
As more CO_2 is added the equilibrium of the reaction is shifted to the right, so the net effect is acidification.

$$CO_3^{2-} + H^+ \rightleftharpoons HCO_3^-$$
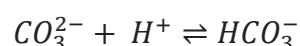
The H^+ ions created by CO_2 hydration react with CO_3^{2-}, reducing their concentration while increasing HCO_3^-. From here on CO_3^{2-} refers to carbonate ions, while carbonates refer to any solid precipitate that forms when carbonate ions react with divalent cations

e.g., $CaCO_3$ and $MgCO_3$. Rather confusingly for non-ocean chemists, the calcification reaction, shown below, is simultaneously a sink for carbon, but also a source of CO_2.

$$Ca^{2+} + 2HCO_3^- \rightleftharpoons CaCO_3 + CO_2 + H_2O$$

Calcification increases CO_2 concentration and decreases alkalinity (HCO_3^-) in the water it occurs in, which in the case of surface waters, reduces the concentration gradient between surface waters and the atmosphere, in turn reducing CO_2 uptake. In contrast, $CaCO_3$ dissolution increases pH of the surrounding medium by removing CO_2 and increasing alkalinity (HCO_3^-). When this occurs in surface waters, this increases the concentration gradient of CO_2 from the atmosphere, allowing the ocean to absorb more CO_2.

Dissolution

Carbonates produced by fish are highly soluble compared to calcite and aragonite produced by calcifying microorganisms. The only known measurement of the solubility of piscine carbonates (in this case produced by Gulf toadfish, *Opsanus* beta) suggests that they are 1.95-fold more soluble than aragonite, and ~3-fold more soluble than calcite (Woosley, Millero and Grosell, 2012). This higher solubility suggests that piscine carbonates will dissolve at shallower depths than those produced by calcifying microorganisms.

The HCO_3^- created by the dissolution of piscine carbonates is proposed to explain at least some of the increase in titratable alkalinity observed within 1,000 m of the surface of the ocean (Wilson *et al.*, 2009). When calcification and dissolution occur in the same area of the ocean, there is no net effect on ocean chemistry, as all changes associated with calcification are reversed by dissolution. In contrast, when there is a spatial separation of calcification and dissolution, HCO_3^- is transported from the area of calcification to the area of dissolution.

Calcification below the surface mixed layer, followed by dissolution near the surface, may have the effect of transporting alkalinity towards the surface of the ocean, where it can enhance the ocean's ability to absorb atmospheric CO_2. This is possible due to the behaviour of fish, many of which undergo diel vertical migration, which generally entails inhabiting shallower waters during the night, and deeper waters during the day (Watanabe *et al.*, 1999). This can be to avoid visual predators during the day, or to track prey species, as many zooplankton also undergo this movement pattern (Hays,

2003). It has been proposed that mesopelagic fish may drive an 'upward alkalinity pump', by producing gut carbonates predominantly during the day (at depth) and excreting these at night (near the surface), where they rapidly dissolve (Roberts *et al.*, 2017). This may have a significant impact on ocean chemistry due to the possibility that mesopelagic fish make up >90% of total marine fish biomass (Irigoien *et al.*, 2014) and that they may produce carbonates that dissolve rapidly in seawater, similar to the carbonates of species that have already been studied (Woosley, Millero and Grosell, 2012; Foran, Weiner and Fine, 2013).

Calcification near the surface, followed by carbonate excretion and dissolution at depth, will reduce the ability of the ocean to absorb CO_2. The likelihood of this is increased by a number of factors. Any carbonates produced by fish that reside entirely within the surface mixed layer, that subsequently sink and dissolve below the surface mixed layer, will reduce surface alkalinity. This is more likely to occur in areas where saturation state for carbonates is high, such as warm water, or areas of already high alkalinity.

Sedimentation/ burial

Carbonates that do not dissolve before sinking to the seafloor can form part of the sediment (Perry *et al.*, 2011). Shallow areas, particularly those with overlying water where the saturation state for piscine carbonates is >1, and containing high fish biomass are likely to contain the greatest proportion of carbonate sediment of piscine origin. Shallow areas usually require carbonates to sink for a shorter period before reaching the seafloor, while high saturation states result in slower, or perhaps a total lack, of dissolution. Areas of high fish biomass will produce a greater number of carbonates available to become sediment. Combining these three factors highlights shallow tropical environments, particularly tropical coral reefs and mangroves (Perry *et al.*, 2011), as areas where significant sedimentation of carbonates may occur.

<u>**Endogenous control of gut carbonate formation**</u>

Fish size

Teleost fish represent an enormous range in body size, from newly hatched larvae weighing <10 mg, to ocean sunfish, *Mola mola*, weighing up to 1,000 kg, a range that covers eight orders of magnitude. While whole animal metabolic rate increases with increasing body mass, the metabolic rate per kg of body mass, known as mass-

specific metabolic rate, decreases (Clarke and Johnston, 1999; Glazier, 2005). In fish, this accounts for a ~1.6-fold increase in mass-specific metabolic rate for each ten-fold decrease in body mass (Clarke and Johnston, 1999; Wilson *et al.*, 2009). As carbonate production rate is tightly linked to metabolic rate, this suggests that many fish, that are equal in total biomass to a single larger fish, will produce more carbonates (Wilson *et al.*, 2009). Smaller fish also face a greater osmoregulatory challenge, with ion turnover increasing ~1.9-fold for each ten-fold decrease in body mass (Grosell, Nielsen and Bianchini, 2002). This equates to an increase in water loss, which must be matched by elevated drinking rate, providing the intestine with more Ca^{2+} for precipitation. Thus, carbonate production rate is more sensitive to body mass than metabolic rate, increasing 2-fold for every 10-fold decrease in body mass (Perry *et al.*, 2011).

Carbonate production rate varies greatly between species in which it has been measured, with calculated carbonate production rates at 25 °C of ~45, ~14 and ~167 µmol/kg/h in sheepshead minnow, Gulf toadfish and European flounder, respectively (Reardon *et al.*, in preparation; Wilson *et al.*, 2009; Heuer *et al.*, 2016). These values are influenced by a multitude of factors (in addition to body mass) that will be covered later in this review, including temperature and salinity. It is important to note that the carbonate production rate given for sheepshead minnow was measured at 25 ppt, and that a much higher rate would be expected at a salinity relevant to the open ocean (Wilson *et al.*, 2009). Carbonate production rates of ~5-100 µmol/kg/h have also been measured in 11 species, at a temperature of ~26 °C and salinity of 36.6 ppt (Perry *et al.*, 2011), emphasising the range of carbonate production rates possible, independent of salinity and temperature. More research is required to help understand what rate of carbonate production may be typical of most marine fish, particularly of understudied mesopelagic species such as myctophids (lanternfish) that may make up the major proportion of global marine fish biomass (Irigoien *et al.*, 2014).

Calcium demand

The Ca^{2+} concentration of seawater contributes to the osmotic stress experienced by fish, as they regulate blood Ca^{2+} concentration to ~15 – 30% that of seawater (Hwang and Lin, 2013). However, fish also require Ca^{2+} throughout their lifetime for processes such as bone and scale growth, as well as neuronal signalling. In addition, extra Ca^{2+} can be required at certain points, such as by females during gonad maturation, in order

to provide oocytes with the substrate for subsequent skeletal development of their offspring.

Oocyte development is regulated by oestrogens (Tyler and Sumpter, 1996). Under freshwater conditions estradiol, an oestrogen, can cause Ca^{2+} resorption from scales, bones and the external medium (Mugiya and Watabe, 1977; Carragher and Sumpter, 1991; Persson, Sundell and Björnsson, 1994). Implantation of one form of estradiol, 17β-estradiol (E2), causes significant elevations of Ca^{2+} in the plasma of sea bream (*Sparus aurata*) (Guerreiro *et al.*, 2004; Guzmán *et al.*, 2004). There are receptors for E2 in the intestine (Socorro *et al.*, 2000; Filby and Tyler, 2005) and as Ca^{2+} is abundant in seawater, absorption from ingested seawater would reduce the need to resorb Ca^{2+} from other tissues, where it performs a useful function to the fish. Any additional absorption of Ca^{2+} from the gut would be likely to reduce carbonate production by the fish in the short term.

Implantation of E2 into seawater-acclimated rainbow trout causes large elevations of Ca^{2+} in both plasma and muscle (Al-Jandal *et al.*, 2011). While this was not directly proven to be via intestinal Ca^{2+} uptake, there was a reduction in $CaCO_3$ excretion rate and no significant difference in intestinal fluid Ca^{2+} concentration, so it seems likely that at least some of the extra Ca^{2+} in tissues was absorbed from the intestine. Indeed, the amount of additional Ca^{2+} stored in tissues would account for 69 % of the observed reduction in carbonate excretion (Al-Jandal *et al.*, 2011).

The added calcium demand from oocyte maturation is unlikely to significantly affect the contribution of fish to marine $CaCO_3$ production, as it only affects females that have undergone sexual maturation, within the appropriate reproductive season. However, this does demonstrate that internal demand for Ca^{2+} is likely to affect the amount of carbonate excreted by individual fish. This does highlight the importance of taking sex (Tannenbaum *et al.*, 2019) and development stage into account when conducting research on carbonate production. Inclusion of females undergoing oocyte maturation into studies on carbonate production may result in underestimations of carbonate production when compared to other individuals. Any other calcium-intensive processes fish undergo may also result in a reduction of gut carbonate production.

Protein regulation of gut carbonate formation

While the intestinal ion transport mechanisms that create the environment suitable for calcification are fairly well understood, the molecular mechanisms regulating the precipitation process itself have only recently been investigated. Like most other biominerals, fish gut carbonates contain an organic matrix, composed at least partially of proteins (Schauer et al., 2016, 2018). These proteins regulate calcification in a dose-dependent manner, with relatively low concentrations (as occur at the start of the intestine) enhancing calcification, while higher concentrations (resulting from sequential water absorption along the length of the intestine) inhibit calcification (Schauer et al., 2016, 2018). As well as effects on rate of calcification, matrix proteins also affect magnesium content of precipitates (Schauer et al., 2016), possibly explaining the diversity in magnesium content and precipitate structure observed between species (Perry et al., 2011; Salter et al., 2019).

The presence of a proteinaceous organic matrix contained within fish gut carbonates is consistent with other sites of eukaryotic biomineralization (Alvares, 2014; Marin & Luquet, 2007; Wang & Nilsen-Hamilton, 2013). Acidic proteins were commonly found in the organic matrix of Gulf toadfish, with ~17% of proteins having an isoelectric point (pI – the pH at which the protein carries no net electrical charge) lower than 5 (Schauer et al., 2016). The presence of acidic proteins is common to proteins involved in biomineralization, particularly calcification (Marin & Luquet, 2007). The acidic domains of matrix proteins are thought to bind cationic Ca^{2+}, and attract further cations to the site of precipitation (Marin & Luquet, 2007). Acidic, hydrophilic peptides may also increase calcification rate by disturbing the structuring of water around the site of mineralization, decreasing the diffusive barrier around the forming mineral, allowing quicker incorporation of ions, particularly calcium, into the growing crystal (Elhadj et al., 2006; Schauer & Grosell, 2017).

As the inorganic chemistry in the intestine heavily favours precipitation, the ability of these proteins to inhibit calcification is important as unchecked calcification could cause intestinal blockage (Schauer et al., 2016). Proteins in the matrix of Gulf toadfish are suspected to have large areas of intrinsic disorder (Schauer et al., 2016), which is characteristic of sites of biomineralization (Marin & Luquet, 2007; Wojtas et al., 2012). These disordered proteins, which lack significant secondary and tertiary structure, are hypothesized to inhibit excessive mineralization, as their flexibility provides a large

area for the protein to bind to mineral step edges, inhibiting further calcification (Gerbaud et al., 2000; Wojtas et al., 2012).

Gulf toadfish (*Opsanus beta*) and European flounder (*Platichthys flesus*) share ~35% of the proteins in the matrix within their precipitates (Schauer et al., 2018). This is despite both species producing high magnesium calcite precipitates with an ellipsoidal morphology (Salter et al., 2012; Woosley et al., 2012). It would be an interesting next step to investigate the composition of the matrix within precipitates of different morphology. Carbonate morphology appears to be more related to the family or species that produced them, than to environmental conditions such as temperature (Salter et al., 2019). If the organic matrix proteins of species that produce similar morphology carbonates are more similar to each other, than to those that produce different carbonate morphologies, this would be convincing evidence that the composition of the organic matrix is key to controlling precipitate mineralogy and morphology.

Environmental effects on carbonate production

The majority of oceanic pelagic calcium carbonate production has long been considered to be dominated by marine microorganisms (Feely *et al.*, 2004). This is at least partially due to carbonate-rich sediments considered as being composed primarily of $CaCO_3$ derived from coccolithophores and foraminifera (Morse, Arvidson and Lüttge, 2007). Coccolithophores make up approximately half of pelagic microbial production through calcification of exoskeletal plates called coccoliths, while pteropods and foraminifera are also significant contributors. Corals, echinoderms, coralline algae and bivalves are benthic calcium carbonate producers with various ecological functions including habitat provisioning. Corals are extensively studied due to their perceived vulnerability to the effects of climate change (Hoegh-Guldberg *et al.*, 2007), but they are a relatively small contributor to global carbonate production due to the small area of ocean they occupy.

Fish-derived calcium carbonate varies significantly from other forms of marine calcium carbonate in two important ways. Unlike the calcium carbonate produced by organisms such as coccolithophores and corals, gut carbonates are a by-product of osmoregulation and calcium homeostasis and are excreted continuously throughout the lifetime of a fish. Fish carbonates undergo precipitation in conditions isolated from

the external environment, where its precipitation is stimulated in order to facilitate water absorption and reduce calcium absorption.

Carbon dioxide

Due to anthropogenic CO_2 emissions, global atmospheric CO_2 concentrations are expected to reach up to 936 µatm by 2100 and up to 1962 µatm by 2200 (IPCC Panel, 2014). Atmospheric CO_2 will equilibrate with surface oceans, reducing their pH. In addition to this, there is significant spatial and temporal variability of ocean pCO_2, with localised areas of high CO_2 including upwelling, intertidal environments, submarine volcanic emissions and midwater oceanic oxygen minimum layers. Indeed, nearshore habitats commonly experience ~5000 µatm fluctuations in CO_2 (Baumann, 2019). High CO_2 is also characteristic of intensive aquaculture, where fish can experience CO_2 well in excess of end of century predictions (Damsgaard *et al.*, 2015; Ellis, Urbina and Wilson, 2017). Particular importance has been attributed to the CO_2 tolerance of fish due to varied effects of CO_2 on growth (Gräns *et al.*, 2014; Stiller *et al.*, 2015; Mota *et al.*, 2019), mineralization (Di Santo, 2019), sensing (Chung *et al.*, 2014; Porteus *et al.*, 2018), immunity (Machado *et al.*, 2020; Mota *et al.*, 2020) and behaviour (Munday *et al.*, 2009; Clark *et al.*, 2020). The amount of CO_2 variability species experience in their habitats is thought to affect their sensitivity to elevated CO_2, with species, or populations, from more variable areas tending to be more tolerant to high CO_2 (Kelly, Padilla-Gamiño and Hofmann, 2013; Müller, Trull and Hallegraeff, 2015; Baumann, 2019).

Due to generation of CO_2 from metabolism, CO_2 in the blood of fish is higher than in the surrounding seawater. Fish excrete metabolic CO_2 down this partial pressure gradient. Under elevated CO_2 conditions, metabolic CO_2 accumulates in the blood until the concentration gradient is restored and excretion resumes. The excess H^+ in the blood caused by elevated CO_2 causes an acidosis. Fish quickly compensate the initial pH drop caused by CO_2 induced acidosis by elevating plasma HCO_3^- (Cameron, 1978; Montgomery *et al.*, 2019). While this restores plasma pH to pre-exposure levels, there is a persistent and proportional elevation in both plasma pCO_2 and HCO_3^- concentration, i.e. if pCO_2 doubles, HCO_3^- concentration must double to restore the original pH. However, although pH regulation is vital for cell function and survival, the resulting elevated levels of CO_2 and HCO_3^- in the blood can exert their own downstream effects on physiology, independent of pH.

Bicarbonate secretion

Elevated CO_2 stimulates base retention (and/or acid excretion) at the gills in order to compensate CO_2 induced acidosis, but also leads to a seemingly counterproductive increase in intestinal bicarbonate secretion (Reardon *et al.*, in preparation; Perry *et al.*, 2010; Heuer, Esbaugh and Grosell, 2012; Heuer *et al.*, 2016; Gregório *et al.*, 2019). This suggests that intestinal bicarbonate secretion is not dynamically adjusted to facilitate acid-base regulation (Brauner *et al.*, 2019). Increased bicarbonate secretion is stimulated by both elevated plasma pCO_2 and HCO_3^- (Grosell *et al.*, 2005).

Intestinal bicarbonate secretion
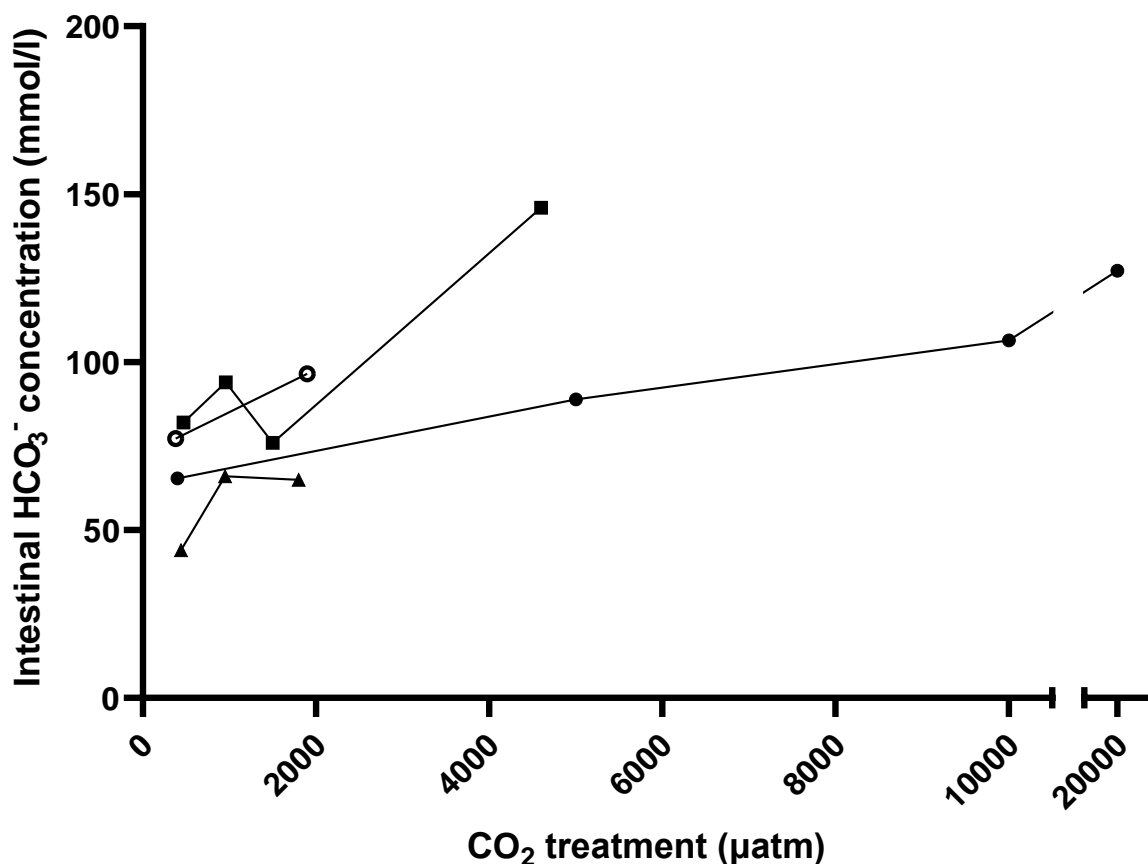
Figure 1: The effect of seawater CO_2 concentration on intestinal HCO_3^- concentration. Filled circles (Gulf toadfish) from Heuer et al., (2016). Unfilled circles (Guld toadfish) from Heuer et al., 2012. Triangles (sea bream) from Gregório et al., 2019. Squares (European flounder) from Reardon et al., (in prep).

A study on flounder *in vivo* at 12 °C found increases in intestinal HCO_3^- concentration only above 1500 µatm (Reardon *et al.*, in preparation), with a ~2-fold increase in intestinal HCO_3^- concentration between 1500 and 4600 µatm, a ~2.1-fold increase in CO_2 (see figure 1 for this and other data mentioned in this paragraph). In contrast, sea

bream at 25 °C show a ~1.5-fold increase in intestinal HCO_3^- concentration between 440 and 950 µatm, a ~2.2-fold increase in CO_2 (Gregório *et al.*, 2019). However, there was no further increase between 950 and 1800 µatm, a ~2-fold increase in CO_2 (Gregório *et al.*, 2019). Sea bass at 16.2 °C increase intestinal HCO_3^- ~2.2-fold in response to ~1700 µatm CO_2, relative to control (~500 µatm), a ~3.4-fold increase in CO_2 (Alves *et al.*, 2020). This is particularly interesting due to the CO_2-associated ~2-fold increase in intestinal fluid volume (Alves *et al.*, 2020), implying a ~4.4-fold increase in intestinal HCO_3^- content, which matches well with observed increases in intestinal carbonate precipitate content (Alves *et al.*, 2020). Plainfin midshipman exhibit a ~1.65-fold increase from control (~400 µatm) to 5% CO_2 (~50,000 µatm), a 125-fold increase in CO_2 (Perry *et al.*, 2010). Gulf toadfish at 22-25 °C show a 1.25-fold increase in intestinal fluid HCO_3^- from 380 to 1900 µatm, a 5-fold increase in CO_2 (Heuer, Esbaugh and Grosell, 2012). Data from the same group on the same species at 22-27 °C showed increases in intestinal HCO_3^- concentration of 1.35-fold, 1.2-fold and 1.2-fold between 400, 5000, 10,000 and 20,000 µatm, respectively, which represent 12.5-fold, 2-fold and 2-fold increases in CO_2 (Heuer *et al.*, 2016). Spot measurements of intestinal HCO_3^- concentration are not necessarily reliable indicators of bicarbonate secretion rate, however. Any increase in calcification associated with elevated HCO_3^- concentration results in the consumption of HCO_3^- to form $CaCO_3$, and production of CO_2, acidifying the intestinal fluid and further reducing HCO_3^- concentration. In addition, changes in drinking rate and fractional fluid absorption can affect the total volume of intestinal fluid, and therefore HCO_3^- concentration in intestinal fluid.

It is possible that the varied methodologies in collecting (rectal collection catheter + whole intestine vs. whole intestine vs. removal of intestinal fluid from live fish) samples to measure intestinal HCO_3^- concentration affected the trends in HCO_3^- concentration observed for different species. However, as even the same methods employed by the same individuals on the same species have shown inconsistency in findings (Heuer, Esbaugh and Grosell, 2012; Heuer *et al.*, 2016), it is difficult to draw a conclusion on how intestinal HCO_3^- concentration will be affected by environmental CO_2 increases. Gulf toadfish and sea bream appear to exhibit a sharp increase in intestinal HCO_3^- concentration up to 1000 µatm, followed by a plateau as CO_2 increases further (Heuer, Esbaugh and Grosell, 2012; Heuer *et al.*, 2016; Gregório *et al.*, 2019). In contrast, European flounder show the opposite, with intestinal HCO_3^- concentration only

responding to increases in CO_2 above 1500 µatm (Reardon *et al.*, in preparation). Whether this is an artifact of sampling and methodology, or if flounder have a different threshold for responding to environmental CO_2 increases, is unclear.
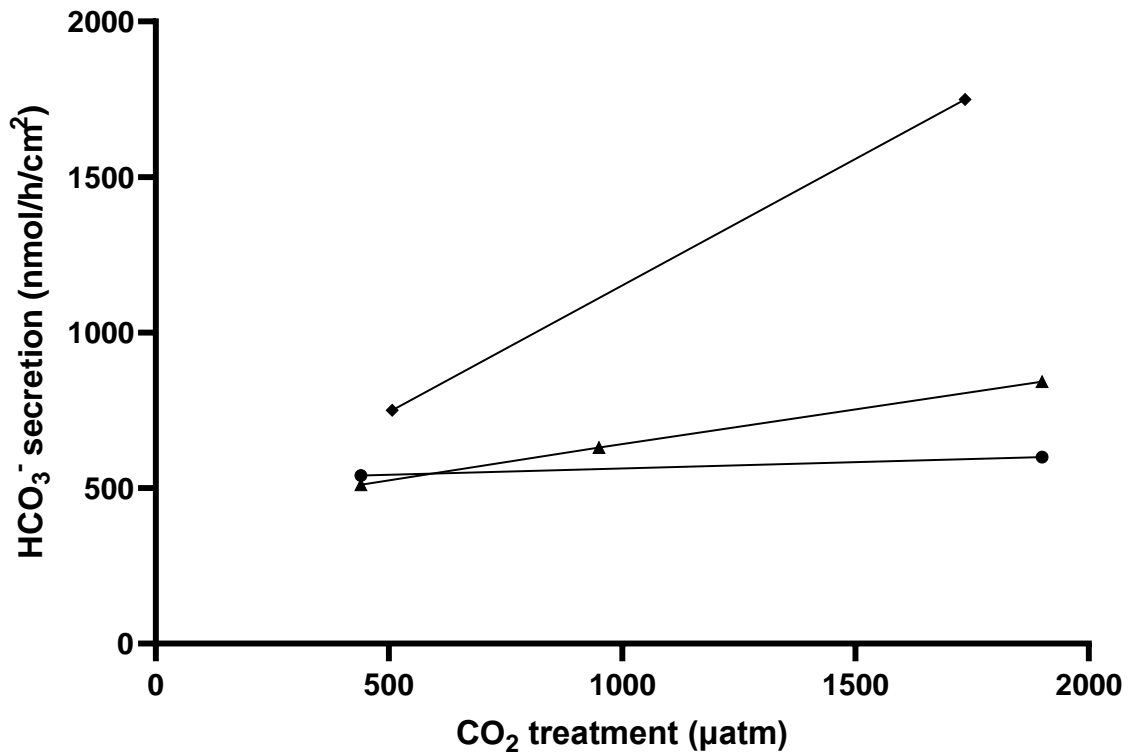
Figure 2: The effect of whole animal acclimation to a range of seawater CO_2 levels on HCO_3^- secretion rate by anterior intestinal tissue, excised and measured in Ussing chambers ex vivo. Circles (Gulf toadfish) from Heuer et al., (2016). Triangles (sea bream) from Gregório et al., 2019. Diamonds (sea bass) from Alves et al., (2020).

Studies measuring anterior intestine HCO_3^- secretion rate *ex vivo* find that prior *in vivo* acclimation to elevated CO_2 versus control (low CO_2) conditions tends to increase intestinal HCO_3^- secretion when the dissected tissues are incubated in the same serosal salines (Heuer and Grosell, 2016; Gregório *et al.*, 2019; Alves *et al.*, 2020). This is true even when the serosal saline has levels of HCO_3^- and CO_2 that mimic control conditions *in vivo* (Heuer and Grosell, 2016; Gregório *et al.*, 2019; Alves *et al.*, 2020). In sea bream at 25 °C, HCO_3^- secretion increases by ~1.65-fold from ~440 to ~1900 µatm, a 4.3-fold increase in CO_2 (Gregório *et al.*, 2019). Proportional increases in HCO_3^- at 950 µatm suggest this is a linear relationship (see fig. 2) and appears to be due to a CO_2-induced upregulation in expression of genes related to ion transport machinery (slc26, slc4, nkcc and aquaporin 1b) (Gregório *et al.*, 2019). The anterior intestine of sea bass appears to be even more sensitive to CO_2 acclimation, exhibiting

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a ~2.3-fold increase in HCO_3^- secretion from ~500 to ~1700 µatm CO_2, a ~3.4-fold increase in CO_2 (Alves *et al.*, 2020). As in sea bream, there was an upregulation in expression of genes related to ion transport machinery, although the degree of upregulation was greater in sea bass, despite a similar increase in CO_2, perhaps explaining the difference in secretion rates (Gregório *et al.*, 2019; Alves *et al.*, 2020). The intestinal tissue of Gulf toadfish at 25 °C respond less strongly to CO_2 acclimation, showing only ~1.1-fold increase in HCO_3^- secretion rate from 440 to 1900 µatm, a ~2.3-fold increase in CO_2 (Heuer and Grosell, 2016). The values for HCO_3^- secretion at 440 µatm were very similar between sea bream and toadfish, despite serosal saline containing ~1.35-fold higher CO_2 and ~1.52-fold higher HCO_3 in the experiments on sea bream tissue (Heuer and Grosell, 2016; Gregório *et al.*, 2019). The difference in responsiveness to CO_2 acclimation is puzzling, but the low responsiveness of toadfish could be explained by aforementioned differences in serosal saline. It is unlikely to be explained by differences in acclimation times of 2-4 weeks for toadfish compared to 3 months for sea bream and 35 days for sea bass (Heuer and Grosell, 2016; Gregório *et al.*, 2019; Alves *et al.*, 2020), which are both long enough for changes in gene expression to be completed. Anterior intestinal gene expression has not been measured for toadfish under high CO_2 conditions, but it is possible that there is a lower degree of upregulation of genes involved in ion transport, compared to that found in sea bream or sea bass.

In addition to elevated HCO_3^- secretion induced by acclimation, serosal saline mimicking blood chemistry changes associated with elevated environmental CO_2 (hereby 'high CO_2 saline') also increase intestinal HCO_3^- secretion in Gulf toadfish (Heuer and Grosell, 2016). This is regardless of whether the intestinal tissue was previously acclimated to high CO_2. In Gulf toadfish, high CO_2 saline increased HCO_3^- secretion by ~1.5-fold (Heuer and Grosell, 2016). As such, it appears that for Gulf toadfish at least, supply of CO_2 and HCO_3^- from the blood exerts stronger control over HCO_3^- secretion than intestinal tissue acclimation. Serosal saline with elevated HCO_3^- concentration has been demonstrated to increase HCO_3^- secretion in the intestine of sea bass, excised from fish not acclimated to high CO_2 (Alves *et al.*, 2019, 2020). Thus, it seems likely that HCO_3^- secretion rates for sea bass and sea bream would be elevated further by high CO_2 saline. This is also supported by greater increases in intestinal HCO_3^- content in high CO_2 acclimated seabass than would be expected from

the effects of CO_2 acclimation on intestinal tissue acclimation alone (Alves *et al.*, 2020).

The limited available evidence suggests that increases in CO_2 conditions up to 2000 µatm are likely to increase intestinal HCO_3^- secretion, through a combination of increased HCO_3^- and CO_2 supply, as well as upregulation of HCO_3^- transport and CO_2 hydration processes. This is likely to lead to elevated concentrations of HCO_3^- in the intestinal fluid, which could affect how much $CaCO_3$ is precipitated and excreted.

<u>Quantity of $CaCO_3$</u>

Increases in intestinal HCO_3^- secretion associated with environmental hypercapnia make available more substrate for calcification. It has therefore been predicted that elevated environmental CO_2 will increase intestinal $CaCO_3$ excretion rates (Heuer and Grosell, 2014). Elevated CO_2 could be expected to affect carbonate production in two main ways. Elevated HCO_3^- secretion could lead to a greater proportion of ingested Ca^{2+} being precipitated (fractional calcium precipitation), leading to an increase in gut carbonate production. It is also possible that elevated intestinal HCO_3^- could lead to a greater proportion of ingested Mg^{2+} being precipitated, which would lead to an increase in total gut carbonate excretion, but also an increase in the Mg:Ca ratio of precipitates. These two processes are not necessarily mutually exclusive and could occur simultaneously.

European flounder at 12 °C show no significant differences in carbonate production up to 1500 ppmv CO_2 , but a 1.9-fold increase in carbonate production from 1500 to 4600 ppmv CO_2, a ~3.1-fold increase in CO_2 (Reardon *et al.*, in preparation) and increases in carbonate excretion rate were strongly correlated with increased intestinal HCO_3^- concentration. In contrast, the opposite effect was observed, with a significant decrease in carbonate production by Gulf toadfish at 20,000 µatm CO_2, compared to control, when measured by collecting carbonates from the tank floor, at 22-27 °C (Heuer *et al.*, 2016). This difference is likely due to increased exposure time of toadfish carbonates (3 vs. 1 day) to the external seawater environment, which is also more corrosive to the carbonates under high CO_2 (low pH) conditions. Interestingly, Gulf toadfish fitted with rectal catheters showed no change in carbonate production when seawater CO_2 was increased from ~400 to ~10,000 µatm, with a non-significant increase in carbonate production of around 25% from ~10,000 to ~20,000 µatm (Heuer

et al., 2016). As rectal catheterisation has previously been shown to produce comparable production rates to tank floor collection when seawater CO_2 and carbonate saturation state is normal (Reardon *et al.*, in prep), the suggestion by Heuer *et al.,* that the difference between the production rates measured by these two methods is due to dissolution (Heuer *et al.*, 2016), is warranted. Gulf toadfish fitted with rectal catheters exposed to 1900 µatm CO_2 showed no significant increase in carbonate production compared to control (380 µatm), a 5-fold increase in CO_2 (Heuer, Esbaugh and Grosell, 2012). The non-significant effects of CO_2 on carbonate production rate in toadfish more closely mirror the lack of significant changes observed in drinking rate, than increased intestinal HCO_3^- concentration (Heuer, Esbaugh and Grosell, 2012; Heuer *et al.*, 2016).

Increases in intestinal carbonate total alkalinity at elevated CO_2 (50,000 µatm) have been observed for the plainfin midshipman when compared to control animals (Perry *et al.*, 2010). In addition, increases in intestinal carbonate abundance, as observed at a single timepoint, have been observed from 440 to 986, with no further increase at 1856 µatm CO_2, in sea bream (Gregório *et al.*, 2019). In sea bass, intestinal carbonate precipitate abundance increased in response to ~1700 µatm CO_2, compared to ~500 µatm (Alves *et al.*, 2019). In sea bream and sea bass, abundance of carbonate precipitates were associated with increases in intestinal HCO_3^- concentration, although changes were significantly greater (~150% increase in carbonate abundance as opposed to ~50% increase in HCO_3^- concentration in sea bream, ~340% as opposed to 120% in sea bass) (Alves *et al.*, 2019; Gregório *et al.*, 2019).

However, due to the stochasticity of carbonate excretion (Salter, Perry and Smith, 2019), removal of carbonates directly from the intestine of fish at a single timepoint is not a reliable indication of production or excretion rate. Environmental conditions can affect other processes such as gut motility (Brijs *et al.*, 2017), which could affect the retention/ excretion of gut contents even if there were no change in production rate. Some individuals have been observed to excrete carbonates at long intervals (Salter, Perry and Smith, 2019), in which case intestinal precipitate content would be more representative of time since last excretion, than of any treatment effect.

The paucity and lack of consensus between scant existing data makes it difficult to draw conclusions about the effect of elevated CO_2 on the amount of calcium carbonate

produced. In terms of fully understanding the mechanism underlying any effect of hypercapnia on gut carbonate excretion rate, it is important to note that only two studies have investigated the effect of CO_2 on drinking rate and carbonate excretion simultaneously (Heuer, Esbaugh and Grosell, 2012; Heuer *et al.*, 2016). The relationship between elevated CO_2 and intestinal bicarbonate content seems to be complex. There may be interspecies variation on threshold at which CO_2 concentration affects intestinal HCO_3^-, as well as the level at which intestinal HCO_3^- becomes saturated. It seems likely that the amount of carbonate produced is more limited by supply of Ca^{2+} in the ingested seawater than intestinal HCO_3^- concentration. Therefore, elevated intestinal HCO_3^- derived from secretion from the blood, or elevated intracellular CO_2 hydration and HCO_3^- secretion, may not significantly affect carbonate production in the absence of increased drinking rate.

Composition of carbonates

Due to increases in intestinal HCO_3^- secretion under elevated environmental CO_2, along with high levels of intestinal Mg^{2+} from ingested seawater, it has been predicted that Mg^{2+} incorporation into carbonates could be increased if intestinal Ca^{2+} became limiting (Heuer, Esbaugh and Grosell, 2012). In contrast, studies have shown no significant effect of environmental CO_2 on magnesium content of excreted carbonates (Reardon *et al.*, in preparation; Heuer *et al.*, 2016). Heuer *et al.* (2012) noted ~1.1-fold increases in carbonate magnesium content from 380 to 1900 µatm CO_2, a 5-fold increase in CO_2, but these differences were found to be insignificant (Heuer, Esbaugh and Grosell, 2012). It therefore appears that seawater CO_2 concentration has minimal or no effect on magnesium content of excreted precipitates for the species and CO_2 concentrations tested.

Fate of carbonates

In addition to the physiological effects already mentioned, the CO_2 concentration of the seawater fish inhabit also affects their dissolution post-excretion. As CO_2 acidifies water as its concentration increases, it reduces pH but more importantly it also reduces Ω for calcium carbonate minerals due to the simultaneous reduction in carbonate ion concentration. For example, an increase of seawater CO_2 from 400 µatm to 1000 µatm results in a decrease in the Ω of aragonite from 1.89 to 0.91 (calculated in CO2Sys v2.3, assuming salinity = 35 ppt, temperature = 15 °C, alkalinity = 2000 µmol/kg). Only one study has measured dissolution of carbonates post-excretion in multiple CO_2

treatments (Heuer *et al.*, 2016). This showed that exposure of excreted carbonates to 20,000 µatm CO_2 seawater for up to 3 days resulted in a significant ~0.5-fold decrease in carbonates, despite evidence from rectal catheters and intestinal spot measurements that carbonate production rate remained constant or even increased (Heuer *et al.*, 2016). This difference can therefore be attributed to increased rate of carbonate dissolution in high CO_2 seawater. Gulf toadfish have been observed to produce carbonates with high magnesium content (~47.9 mole% $MgCO_3$) (Heuer, Esbaugh and Grosell, 2012), so these carbonates may be particularly vulnerable to dissolution and even more so under high CO_2 conditions. As such, the many species which produce carbonates with less $MgCO_3$ (Salter, Perry and Wilson, 2014; Salter, Perry and Smith, 2019) may show less dissolution under high CO_2 conditions. The corrosiveness of high CO_2 water to carbonates also makes the study of carbonate production at high CO_2 more difficult, as more excreted products are likely to dissolve at elevated CO_2 prior to being collected and analysed, potentially masking increases in their excretion rate (Heuer *et al.*, 2016).

Temperature

Ocean temperature varies widely, with the surface ocean anywhere from -2 °C to 36 °C, with an average of ~15-17 °C. Beneath the surface layer seawater temperature gradually decreases with depth to around 2,000 m, below which seawater is generally quite stable at around 0 °C to 4 °C. Fish inhabit almost all of the ocean, with a theoretical maximum depth of 8,200 m (Yancey *et al.*, 2014). As already mentioned, anthropogenic CO_2 production has the direct effect of CO_2 dissolution in the ocean, resulting in ocean acidification. In addition, a secondary effect of CO_2 in the atmosphere is the greenhouse effect and, thus, climate change. Anthropogenic climate change is expected to increase sea surface temperature by 1 to 3 °C by the end of the century (IPCC Panel, 2014). As such, it is important to understand how carbonate production is affected by temperature.

Most teleost fish are poikilotherms, therefore all bodily processes occur at or close to the environmental temperature (Block *et al.*, 1993). Temperature has a diverse suite of effects on fish physiology, including but not limited to metabolic rate (Gräns *et al.*, 2014; Hvas *et al.*, 2018), aerobic scope (Gräns *et al.*, 2014; Hvas *et al.*, 2018), growth (Gräns *et al.*, 2014; Islam *et al.*, 2020), swimming performance (Norin *et al.*, 2019) and

gut transit time (Miegel *et al.*, 2010). This section will concentrate on the effects of temperature that are relevant to carbonate production.

Physiological processes relevant to carbonate production

Two important effects of elevated temperature on fish physiology, with respect to the production of calcium carbonate, are increases in metabolic and drinking rates (Reardon *et al.*, in preparation; Wilson *et al.*, 2009). The sensitivity of the rate of a biological reaction to temperature is described by a temperature quotient (Q_{10}). A Q_{10} describes by what factor a rate of reaction will change by, due to a 10 °C increase in temperature. For example, a Q_{10} of 1 refers to a reaction that is not sensitive to temperature, while a Q_{10} of 2.5 means that the rate of reaction would be two and a half times faster at 20 °C than at 10 °C.

The average Q_{10} for metabolic rate in fish, calculated from data on 69 species across a 0 to 30 °C temperature range, is 1.83 (Clarke and Johnston, 1999). Elevated metabolic rate increases CO_2 production in all tissues, thereby directly stimulating intestinal HCO_3^- secretion (Wilson and Grosell, 2003; Heuer and Grosell, 2014). Drinking rate increases more at elevated temperatures than would be expected from increases in metabolic rate alone (Reardon *et al.*, in preparation; Evans, Motais and Romeu, 1972; Clarke and Johnston, 1999; Takei and Tsukada, 2001), and in doing so provides more Ca^{2+} for precipitation, in addition to stimulating intestinal HCO_3^- secretion (Wilson and Grosell, 2003).

Calcium carbonate is less soluble at higher temperatures (Plummer and Busenberg, 1982; Morse, Arvidson and Lüttge, 2007), therefore favouring its precipitation in the gut as temperature increases (as well as favouring its preservation in seawater once excreted). This, in combination with the effects of temperature on metabolic rate and drinking rate, are thought to be the key factors in explaining the sensitivity of carbonate production to temperature (Reardon *et al.*, in preparation). Temperature-associated changes in gut residence time may also be important with respect to the crystal structure of excreted carbonates (Miegel *et al.*, 2010; Salter, Perry and Smith, 2019).

Quantity of $CaCO_3$

All available studies support that elevated temperature increases the rate of calcium carbonate excretion (Reardon *et al.*, in preparation; Wilson *et al.*, 2009; Heuer *et al.*, 2016). This is expected, as carbonate production is tightly linked to metabolic rate

(Wilson *et al.*, 2009). A carbonate production Q_{10} of 2.33, 2.94 and 3.21 have been calculated for sheepshead minnow, European flounder and Gulf toadfish, respectively (Reardon *et al.*, in preparation; Wilson *et al.*, 2009; Heuer *et al.*, 2016). Interestingly, all the aforementioned carbonate production Q_{10}s are above the average Q_{10} of 1.83 for metabolic rate across all fish species surveyed (Clarke and Johnston, 1999). This supports the idea that temperature-associated changes in drinking rate and carbonate Ω in the gut increase carbonate production more than would be expected by elevated metabolic rate alone. Unfortunately, no studies have measured metabolic rate and carbonate production rate side-by-side at a range of temperatures, so this cannot be directly confirmed. However, it seems likely that these two factors, probably with the additional Ca^{2+} provided by higher drinking rates playing a key role, contribute to increased carbonate production at elevated temperature.

Intriguingly, data from species in which carbonate production rate has been measured at 4 or more temperatures suggest that carbonate production may not be truly exponential as expected for physiological rate functions generally, and instead may more closely follow the relationship of temperature and metabolic rate shown by Gräns, with a relatively constant carbonate production rate within the middle range of temperatures they tolerate (Gräns *et al.*, 2014). This would suggest that species experiencing only this middle range of their thermal tolerance may exhibit only small increases in carbonate production in response to climate change. In contrast, those living close to their thermal tolerance limits may more dramatically increase their carbonate production as temperature rises.

Composition of carbonates

Elevated temperature linearly increases the incorporation of magnesium into biogenic and abiotic Mg-calcite (Mucci, 1987; Halfar *et al.*, 2000; Dickson, 2002; Morse, Arvidson and Lüttge, 2007). In contrast, temperature changes above 10 °C appear to have little effect on incorporation of magnesium, carbonate morphology or mineralogy, in many species (Salter, Perry and Smith, 2019). An exception is the Scorpaenidae (scorpionfishes), whose carbonates contain a small but significant amount less magnesium at 10 °C than at 25 °C (Salter, Perry and Smith, 2019). In European flounder carbonates, magnesium content sharply increases from 10 to 22% between 7 and 12 °C, before plateauing at ~26% above 19 °C (Reardon *et al.*, in preparation).

It appears that magnesium incorporation into carbonates is relatively stable above 10 to 15 °C. However, if the high sensitivity of magnesium content to temperature found for flounder below 12 °C (Reardon *et al.*, in preparation) holds true for other species, relatively small increases in temperature associated with climate change could cause dramatic increases in magnesium content for cold water species. This is particularly important as temperature sharply drops with increasing depth below the surface layer, where the majority of marine teleosts (mesopelagics) spend a significant portion of their time during their diel vertical migrations (Irigoien *et al.*, 2014).

As elevated magnesium content increases solubility (Morse, Arvidson and Lüttge, 2007), the available evidence suggests that some fish exposed to elevated ocean temperatures will produce carbonates that are more soluble . This contrasts with the concurrent increase in $CaCO_3$ saturation states at elevated temperatures, so it is unclear what the net effect of increased temperature on the actual dissolution of fish gut carbonates would be. This phenomenon may contribute to an overestimate of the effect of temperature on carbonate production when measured experimentally, as the already smaller amounts of carbonate produced at lower temperatures will likely dissolve more quickly when compared to those produced at higher temperatures. Alternatively, the opposite could be true if lower solubility caused by reduced Mg^{2+} content outweighs temperature-associated reduced saturation state.

Other influences of temperature relevant to carbonate production

While fish exposed to higher temperatures consistently produce more carbonates, whether this will affect total oceanic carbonate production by fish is complicated by the ability of fish to shift their distributions to match their thermal tolerance (Sunday, Bates and Dulvy, 2012). Indeed, fisheries in most of the world's coastal and shelf areas now exhibit a higher proportion of species with higher thermal optima than they did in 1970 (Cheung, Watson and Pauly, 2013), suggesting poleward shifts in fish distribution. However, not all species are able to disperse to areas that become thermally appropriate, particularly species that require specific habitats at specific depths (Rutterford *et al.*, 2015). If biomass remains relatively constant, the net effect of elevated temperature will be an increase in carbonate production by fish, regardless of range shifts.

Fish that are exposed to elevated temperatures exhibit decreased maximum size, with a 5% reduction in length per 1 °C increase in maximum sea surface temperature (van Rijn *et al.*, 2017). This is particularly pronounced in active species (10% per 1 °C), which are thought to be important contributors to calcium carbonate production (Wilson *et al.*, 2009). In addition, maximum body mass of fish globally is expected to decline by up to 24% by 2050 (Cheung *et al.*, 2013). The gill-oxygen limitation theory explains this by proposing that the surface area of the gills is unable to keep up with oxygen demand at higher temperatures (Pauly and Cheung, 2018), although this appears not to be the case for most species in which gill surface area and metabolic rate have been measured at a range of temperatures (Scheuffele, Jutfelt and Clark, 2021). Regardless of the mechanism, it appears that marine fish will become smaller as the environment warms (Sandström, Neuman and Thoresson, 1995; Daufresne, Lengfellner and Sommer, 2009). Decreased maximum fish size, if accompanied by increased numbers of smaller fish to make up equal biomass, would increase total carbonate production by fish. This is due to metabolism and carbonate production scaling inversely with body size (Clarke and Johnston, 1999; Wilson *et al.*, 2009). However, models suggest that total marine biomass will decrease due to climate change, which is amplified at higher trophic levels, of which fish form a part (Lotze *et al.*, 2019).

Conclusions:

Due to the intricacies of fish behaviour, life history, maximum/ average size, dispersal capabilities and more, it is extremely difficult to predict how future climate warming will affect $CaCO_3$ production by fish. On balance, it is likely that elevated temperatures associated with climate change will increase the contribution to global carbonate production by fish. This will be accompanied by poleward shifts in carbonate production (Lotze *et al.*, 2019), where carbonates may also dissolve more quickly, if temperature-induced increases in magnesium content outweigh $CaCO_3$'s higher stability in warmer seawater. However, it is as yet unclear how these various conflicting factors will interact to influence carbonate production by fish.

Salinity

Average global ocean salinity is 35 psu, although there is significant spatial variation between 30 and 40 ppt (Antonov *et al.*, 2006; Vine *et al.*, 2020). In addition, areas of freshwater input constitute environments where a salinity gradient from fresh to full

strength salt water exists. Climate change is predicted to affect the global hydrological cycle by increasing precipitation in areas of already low salinity, while increasing evaporation in areas of high salinity, elevating spatial variability in salinity (Melzer and Subrahmanyam, 2017). As teleost fish inhabit all areas of the ocean down to a depth of ~8,400 m (Yancey *et al.*, 2014), it is important to understand how carbonate production can be affected by the range of salinities they encounter.

As a primary role of gut carbonate production is osmoregulation (Wilson, Wilson and Grosell, 2002; Wilson and Grosell, 2003), environmental salinity is likely to have a major influence on their excretion rate. Fish drink seawater to offset the passive water loss and ion gain (osmotic stress) from living in a hyperosmotic environment. As environmental salinity increases, the osmotic gradient from fish to environment increases, so these passive processes occur more rapidly. Fish must therefore drink more seawater, increase the proportion of water absorbed from ingested seawater (increase fractional fluid absorption), or a combination of the two, to compensate for osmotic losses.

Under conditions of 30-35 ppt, fractional fluid absorption has been recorded from 39 to 85% (Shehadeh and Gordon, 1969; Wilson *et al.*, 1996; Wilson, Wilson and Grosell, 2002). In the upper portion of this range there is little scope to further increase fractional fluid absorption to offset the osmotic challenge of elevated salinity. Indeed, fractional fluid absorption by Gulf toadfish decreases from ~69% to ~61% between 35 ppt and 50 ppt, respectively, suggesting that a maximum level of fluid absorption has been reached (Genz, Taylor and Grosell, 2008). This is because the intestine is impermeable to divalent ions, so as monovalent ions and water are absorbed from the intestinal lumen, concentrations of ingested ions such as Ca^{2+}, Mg^{2+}, and SO_4^{2-} (and secreted ions such as HCO_3^-) become more concentrated, and can become limiting to water absorption (Genz, Taylor and Grosell, 2008). It is therefore unlikely that a significant increase in fractional fluid absorption could play a role in adapting to elevated salinity in species with already high fractional fluid absorption. Conversely, it is possible that fractional fluid absorption could become lower under decreased salinity, particularly in the short term if fish are slow to adjust their drinking rate to reflect external osmolality.

Drinking rate

As drinking rate and salinity of ingested seawater together control the supply of Ca^{2+} to the intestine, it is important to understand how changes in salinity affect drinking rate. It is well established that elevated salinity increases drinking rates in teleost fish (Shehadeh and Gordon, 1969; Alves *et al.*, 2020). Elevated salinity causes ingested seawater to be more concentrated in all ions, as well as causing additional osmotic stress, so if drinking rate increases, the number of ions consumed increases exponentially. A salinity increase from ~35 ppt to ≥60 ppt appears to increase drinking rate by the same factor as it controls osmotic gradient (Maetz and Skadhauge, 1968), or by >60% of the increase in osmotic gradient (i.e., if osmotic gradient increases by 50%, drinking rate increases by >30%) (Sardella *et al.*, 2004; Gonzalez, Cooper and Head, 2005). In contrast, increases in salinity from isosmotic (~10 ppt) up to ~55 ppt tend to increase drinking rate by <60% the increase in osmotic gradient (Shehadeh and Gordon, 1969; Tytler and Ireland, 1994; Webb *et al.*, 2001; Sardella *et al.*, 2004; Varsamos *et al.*, 2004). Here we will specifically look at drinking rates of species exposed to a range of salinities, for which bicarbonate secretion and/ or carbonate production have also been measured.

Goldsinny wrasse (*Ctenolabrus rupestris*) and Shanny (*Lipophrys pholis*) at 15 °C exhibit a ~1.2-fold and ~1.3-fold increase in drinking rate between 30 and 35 ppt, respectively, and a further ~2.4-fold and ~1.3-fold increase between 35 and 40 ppt, respectively (Stephens, Perry and Wilson, in preparation). The ~2.4-fold increase in drinking rate observed in goldsinny wrasse is unusually high, as this increase was significantly higher than the increase in osmotic gradient associated with salinity, which has not been observed in other species (Stephens, Perry and Wilson, in preparation). Gulf toadfish at 22-26 °C increase drinking rate by ~1.5-fold from 35 to 50 ppt (Genz, Taylor and Grosell, 2008). Sea bream larvae at 20 °C show a strong positive correlation between drinking rate and salinity, although no significant differences in drinking rate were observed between 35.5 and 55 ppt (Guerreiro *et al.*, 2004).

The salinity tolerance of fish may be important for understanding the response of their drinking rate to changes in salinity. Research carried out by the same groups on relatively stenohaline and euryhaline species has revealed that drinking rate is much more sensitive to salinity in stenohaline species (Stephens, Perry and Wilson, in

preparation; Tytler and Blaxter, 1988). For example, an increase in salinity from 16 ppt to 32 ppt resulted in a 2.7-fold increase in the stenohaline cod (*Gadus morhua*), in contrast to a 1.4-fold increase in the euryhaline plaice (*Pleuronectes platessa*) (Tytler and Blaxter, 1988). This is similar to the differences in measured drinking between the stenohaline goldsinny wrasse and shanny mentioned above (Stephens, Perry and Wilson, in preparation). It is possible that stenohaline species lack the ability to adapt physiological mechanisms to adjust water fluxes, so must rely on changes in drinking rate to cope with salinity stress to a greater degree than euryhaline fish. This is important to note as, due to the nature of studies using salinity as an experimental variable, euryhaline fish are unsurprisingly the most commonly used study species. However, it may be misleading to draw conclusions about fish in general from this, as ~99% of marine fish are stenohaline (Whittamore, 2012).

Bicarbonate secretion

Elevated concentrations of Cl^- in seawater associated with elevated salinity need to be absorbed in order to facilitate water absorption. As HCO_3^-/Cl^- exchange forms an important part of water absorption, this process would be expected to be upregulated under conditions of elevated salinity (unless all additional Cl^- provided by elevated salinity is absorbed prior to reaching the intestine), leading to elevated HCO_3^- secretion and elevated HCO_3^- concentration in the intestinal lumen.

The anterior intestine of sea bream at 21 °C exhibit a ~1.6-fold increase in HCO_3^- secretion rate when acclimated to 55 ppt compared to 35 ppt, measured *ex vivo* in Ussing chambers (Gregório *et al.*, 2013). This increase in HCO_3^- secretion rate is close to the increase in ion concentrations in seawater between 35 and 55 ppt, i.e., ~1.6-fold.

Gulf toadfish at 22-26 °C show no significant differences in the *in vivo* intestinal HCO_3^- concentration at 9, 25 and 50 ppt (Genz, Taylor and Grosell, 2008). Similarly, sea bream exhibit no significant difference in intestinal HCO_3^- concentration *in vivo* between 35 and 55 ppt, although there is a statistically significant trend towards increased HCO_3^- concentration from 12 to 55 ppt (Gregório *et al.*, 2013). The maintenance or increases in intestinal HCO_3^- concentration in these species despite increased drinking rate or intestinal fluid volume implies greater rates of HCO_3^- secretion.

Carbonate production

Elevated concentrations of Ca^{2+} and HCO_3^- in the intestinal lumen, from elevated concentrations in the ingested seawater and elevated HCO_3^- secretion respectively, are the two substrates for $CaCO_3$ precipitation. Therefore, as long as one of these is increased with increasing salinity, and together they are sufficient to exceed saturation state, intestinal $CaCO_3$ production would be expected to increase. As drinking rate and HCO_3^- secretion both appear to increase with increasing salinity, the effect on intestinal carbonate production is likely to be significant.

Goldsinny wrasse at 15 °C excrete 5.1-fold more $CaCO_3$ at 35 than 30 ppt, and a further 2.3-fold more at 40 compared to 35 ppt (Stephens, Perry and Wilson, in preparation). This represents fractional calcium precipitation (as a % of ingested Ca^{2+}) of 8% at 30 ppt, compared to ~30% at 35 and 40 ppt (Stephens, Perry and Wilson, in preparation). Increases in $CaCO_3$ excretion from goldsinny wrasse above 35 ppt can therefore be solely attributed to increase in Ca^{2+} supply via elevated drinking rate. Goldsinny wrasse are a relatively stenohaline species, in contrast to the generally euryhaline species which have been used to study the response of fish to salinity stress. This is a key avenue for future research to better understand how a variety of fish respond to ocean-relevant variations in salinity.

Shanny at 15 °C also increased carbonate excretion rate at higher salinities, with a ~1.4-fold increase from 30 to 35 ppt, and a ~1.6-fold increase from 35 to 40 ppt (Stephens, Perry and Wilson, in preparation). Strangely, shanny appeared able to precipitate ~2-fold more Ca^{2+} than would be available based on their drinking rate alone. This suggests another source of Ca^{2+}, such as uptake at the gills, followed by secretion into the intestinal fluid from the blood, in addition to that from ingested seawater (Stephens, Perry and Wilson, in preparation). Despite the impermeability of the intestine to divalent ions, there is a precedent for intestinal calcium secretion by a combination of parathyroid hormone-related protein and stanniocalcin 1 (Fuentes, Power and Canário, 2010). Alternatively, this could be related to potential inaccuracies in measuring drinking rate. Specifically, the long time-scale (days) of measuring carbonate production should provide an accurate estimate of the daily average, whereas drinking rate (measured over just a few hours) could potentially mis-represent the daily average if the drinking process is relatively stochastic or has substantial diel variability.

Gulf toadfish produce ~9-fold more $CaCO_3$ at 50 compared to 35 ppt (Genz, Taylor and Grosell, 2008). While salinities this extreme are rare in the natural environment, this does exemplify that another species' carbonate production rate is sensitive to salinity. Obviously smaller increases in carbonate production would be expected with smaller, more environmentally relevant, increases in salinity, but the relative change in gut carbonate production at smaller salinity intervals is yet to be fully characterised across a wide range of species.

The intestinal carbonate content of sea bream appears to correlate positively with salinity from 12 to 55 ppt, measured by the dry weight of all carbonate aggregates present in the intestine when dissected (Gregório et al., 2013). The dry weight of precipitates contained in the intestine of Japanese eels, Anguilla japonica, increased from ~10.5 ppt to 35 ppt, with a noticeable exponential trend due to the greater difference in carbonate content between 21 ppt and 35 ppt, than between 10.5 and 21 ppt (Mekuchi, Hatta and Kaneko, 2010). However, these values include the dry weight of organic mucus associated with carbonates, which could be independently affected by salinity. Double-endpoint titration measures CO_3^{2-} content of precipitates more accurately and is unaffected by the ratio of calcium to magnesium present in the precipitates. In addition, measurement of carbonate content of the gut at a given time point is an unreliable indicator of carbonate production rate, for reasons mentioned in the section on CO_2.

Carbonate composition

Incorporation of magnesium into carbonates could be affected by elevated salinity, as the magnesium content of ingested seawater increases in proportion with salinity. Also, precipitation of a higher proportion of ingested magnesium at higher salinity might occur, if calcium precipitation were also maintained or elevated, as this would allow for more water absorption. This could be necessary to maintain the osmotic gradient required for water absorption when drinking elevated salinity seawater, as the elevated concentration of Mg^{2+} might inhibit water absorption. However, no significant differences were observed for magnesium content of precipitates in shanny exposed to 30 – 40 ppt, averaging 25% Mg^{2+} (Stephens, Perry and Wilson, in preparation). In contrast, there was a noticeable decline in magnesium content of precipitates produced by goldsinny wrasse from 35 to 40 ppt, from ~48% to ~16 % Mg^{2+} respectively (Stephens, Perry and Wilson, in preparation). This likely due to the ~2.4-

fold increase in drinking rate at this salinity, which makes available more Ca^{2+} for precipitation. Further research into the effect of salinity on Mg^{2+} content of precipitates is key, as Mg^{2+} content of precipitates affects their solubility, which could therefore affect the inorganic carbon cycle. However, as climate change related salinity modifications are likely to be small, it is unlikely that they will have a large effect on Mg^{2+} content of carbonates produced by fish.

Conclusions

All evidence suggests that fish produce more $CaCO_3$ at higher salinities. Notably, this is likely a non-linear relationship, with the difference in carbonate production caused by a 1 ppt increase being greater than the difference caused by a 1 ppt decrease. This is particularly important due to predictions that climate change will elevate spatial salinity variability, as the increases in carbonate production in areas of high and increasing salinity are likely to outweigh losses in carbonate production in areas of low and decreasing salinity. As such, differences in carbonate production due to salinity are likely to be exaggerated, but the overall contribution of fish to oceanic $CaCO_3$ production due to salinity is likely to increase. Research on stenohaline marine species is a key avenue for further research, as these fish dominate marine biomass and may be more sensitive to small changes in salinity than better studied euryhaline species.

Oxygen

Like all other eukaryotes, fish rely upon a supply of oxygen to live. Atmospheric O_2 is not expected to be significantly affected by anthropogenic climate change, where areas of low O_2 (hypoxia) are generally restricted to high altitudes, and therefore away from sea level. In contrast, areas of hypoxia are already common in marine and freshwater environments. Hypoxia has varied effects on fish, including behaviour (Kramer and McClure, 1982; Domenici, Steffensen and Marras, 2017), reproduction (Wu *et al.*, 2003), development (Shang and Wu, 2004), thermal tolerance (Motyka *et al.*, 2017; Zanuzzo *et al.*, 2019), mitochondrial physiology (Du *et al.*, 2016; Martos-Sitcha *et al.*, 2017), digestion (Eliason and Farrell, 2014), growth (Chabot and Dutil, 1999; Hrycik, Almeida and Höök, 2017) and reactive oxygen species homeostasis (Du *et al.*, 2016; Borowiec and Scott, 2020). Climate change is expected to increase the severity and frequency of hypoxic events (Diaz and Rosenberg, 2008; Altieri and Gedan, 2015; Breitburg *et al.*, 2018).

Most fish maintain a stable oxygen consumption rate (MO_2) as oxygen concentration (PO_2) declines, until a critical threshold (P_{crit}) is reached, after which MO_2 decreases with decreasing PO_2 (Richards, 2009). In order to extract O_2 from low PO_2 water, fish invoke the hypoxic ventilatory response (HVR) (Perry, Jonz and Gilmour, 2009). The HVR refers to an increase in ventilation volume, driven by elevated ventilatory frequency and/or amplitude (Perry, Jonz and Gilmour, 2009). The increased volume of water flowing over the gills allows for the maintenance of O_2 uptake in hypoxic conditions.

As well as their role in gas exchange, the gills also play an important role in ion regulation. In addition to facilitating oxygen uptake from hypoxic water, the HVR also increases the osmotic stress experienced by fish in hypo- or hyperosmotic water. In marine fish, passive loss of water and gain of ions are directly proportional to gill ventilation (Gonzalez and McDonald, 1992; Wood *et al.*, 2019). In order to compensate for this loss of water at the gills, marine fish experiencing hypoxia must absorb a greater volume of water in the intestine. In this way, hypoxia exhibits a similar osmotic challenge to elevated salinity, but without the need to consume a more concentrated medium to compensate for osmotic water losses. The osmotic stress of hypoxia is exemplified by the effect of combined hypoxia and high salinity on plasma osmolality in coho salmon, which is absent at lower salinities or in normoxia (Damsgaard *et al.*, 2020).

In order to absorb more water from the intestine, fish could increase fractional fluid absorption, increase drinking rate, or a combination of the two. In normoxic conditions, fractional fluid absorption in the intestine varies, from ~39% to 85% (Wilson *et al.*, 1996; Wilson, Wilson and Grosell, 2002). There is no known data on fractional fluid absorption in hypoxia, but fish may be able to increase fractional fluid absorption under hypoxia to a greater degree than they can under elevated salinity. This is because hypoxia has no effect on the ion concentrations of ingested seawater, unlike elevated salinity. Despite this, fractional fluid absorption is still limited by accumulation of divalent ions such as SO_4^{2-} and Mg^{2+} (Genz, Taylor and Grosell, 2008). An increase in fractional fluid absorption could result in an increase in carbonate production, if a greater proportion of ingested Ca^{2+} and Mg^{2+} are precipitated (fractional calcium and magnesium precipitation) in order to aid water absorption.

On the other hand, increases in drinking rate associated with hypoxia are well documented (Marshall and Grosell, 2006; Wood *et al.*, 2019). In order to maintain fractional fluid absorption from the elevated volume of water ingested due to exposure to hypoxia, more Ca^{2+} and Mg^{2+} must be precipitated out of solution. As hypoxia suppresses growth (Chabot and Dutil, 1999; Hrycik, Almeida and Höök, 2017) and reproduction (Wu *et al.*, 2003), the requirements of the fish for Ca^{2+} may be reduced, leading to less uptake from the intestine, and more Ca^{2+} being available for precipitation. Due to these factors, carbonate excretion by fish would be expected to increase with intensifying hypoxia as far as P_{crit} (hereafter termed mild hypoxia).

The effect of hypoxia below P_{crit} (hereafter termed severe hypoxia) on carbonate production is more complex. Ventilation, and therefore the associated osmotic stress, decreases around and below P_{crit} (Perry, Jonz and Gilmour, 2009). In addition, all tested species of fish exhibit the ability to downregulate metabolic rate in response to <10% air saturation (Richards, 2009). This can be by as much as 70% in European eel (Van Ginneken *et al.*, 2001). Reductions in ventilation and metabolic rate would reduce the amount of water required to be absorbed by the intestine. In addition, blood acidification associated with severe hypoxia (Aboagye and Allen, 2018) should reduce the delivery of HCO_3^- to the intestine that stimulates HCO_3^- secretion. Therefore, severe hypoxia or anoxia may result in a reduction in carbonate excretion by fish, in contrast to the increases associated with mild hypoxia. However, fish are likely to avoid areas of severe hypoxia or anoxia due to the detrimental physiological consequences associated with residing in these areas. For these reasons, areas of severe hypoxia and anoxia are likely to have very low rates of carbonate production by fish, but also likely to have an insignificant effect on global fish carbonate production in the ocean.

To my knowledge, only a single study has investigated the effect of hypoxia on gut carbonate production, which exposed European flounder to hypoxic seawater of 50% air saturation (Rogers, 2015). This resulted in a 2.4-fold increase in ventilation volume and therefore increased water loss and ion gain across the gills. This was not accompanied by a measured increase in drinking rate, but the authors suggest that this may be related to the stochasticity of drinking in flounder, or methodological issues related to measurement of drinking rate mentioned previously. Despite lack of effect on drinking rate, 50% hypoxia resulted in a 2.4-fold increase in carbonate excretion relative to control, which precisely matches the increase in ventilation volume. It is

possible that this increase in carbonate excretion could have been met by an increase in fractional calcium precipitation alone, as if 85% of Ca^{2+} was precipitated under hypoxia (the highest recorded fractional calcium precipitation (Wilson *et al.*, 1996)), ~35% of Ca^{2+} would have been precipitated under normoxia, which is within recorded levels (Genz, Taylor and Grosell, 2008).

Conclusions

It appears that areas of mild hypoxia in the ocean may have led to underestimates of the current contribution to global carbonate production by fish (Wilson *et al.*, 2009). This is because increases in gill ventilation associated with the HVR will lead to a larger demand for water absorption from the gut and therefore increased carbonate production. While available evidence is limited, it appears that carbonate production will increase in response to hypoxia, by a factor similar to that which ventilation volume increases by. The effect of severe hypoxia and anoxia are more difficult to predict, and there is no evidence to suggest how carbonate production is affected under these conditions. As hypoxia events increase with climate change, it is likely that fish will produce more carbonates, as more fish will need to invoke the HVR to maintain oxygen consumption under mild hypoxia.

Individual ion concentrations

Calcium and magnesium

As Ca^{2+} and Mg^{2+} are the cationic substrates for calcification, it is intuitive that their concentration in ingested seawater will regulate carbonate production by fish. Indeed, it has been proposed that carbonate precipitation in the gut is important in protecting the kidney from renal stone formation, associated with high Ca^{2+} concentration in urine, in addition to its role in osmoregulation (Wilson and Grosell, 2003). Despite Mg^{2+} being 5-fold higher in seawater than Ca^{2+}, very few carbonates have been observed with >83% Mg^{2+} concentration (Salter *et al.*, 2018). This suggests that Ca^{2+} is preferentially precipitated and may control carbonate production to a greater degree than Mg^{2+}. It is therefore predicted that elevated Ca^{2+} concentration in seawater will increase gut carbonate production, while the effect of Mg^{2+} is more complicated. Early experiments on 17.5 ppt seawater-acclimated rainbow trout showed that a 5-fold increase in water Ca^{2+} concentration resulted in a 5-fold increase in carbonates produced (Shehadeh and Gordon, 1969).

The expression of calcium-sensing receptors (CaSR) has been detected in the intestine of sea bream, with particularly high expression in the anterior intestine, its expression mirroring luminal Ca^{2+} concentration (Gregório and Fuentes, 2018). Treatment of intestinal tissues, mounted in Ussing chambers, with salines of different Ca^{2+} concentration resulted in roughly proportional changes in HCO_3^- secretion (Gregório and Fuentes, 2018). Perfusion of the intestine of flounder with saline with 4-fold higher Ca^{2+} concentration, balanced by an equimolar decrease in Mg^{2+}, results in a ~1.5-fold increase in HCO_3^- secretion, and a ~7-fold increase carbonate excretion rate (Wilson, Wilson and Grosell, 2002). In addition, exposure of isolated intestinal segments to elevated Ca^{2+} concentration resulted in a ~1.5-fold increase in HCO_3^- secretion, while exposure to an equimolar increase in Mg^{2+} concentration did not affect HCO_3^- secretion (Wilson, Wilson and Grosell, 2002). Further *in vivo* experiments on European flounder have shown a similar relationship between perfusion of the intestine with Ca^{2+} and carbonate production, with roughly proportional increases in carbonate production from 10 mM to 40 mM and 90 mM Ca^{2+} (Whittamore, Cooper and Wilson, 2010). Exposure of Japanese eels independently to low-Ca^{2+} and low-Mg^{2+} seawater (both 90% lower than their concentration in seawater – reduction from ~10 mM – ~1 mM Ca^{2+}, or ~50 mM – ~5 mM Mg^{2+} (Hwang and Lin, 2013)) results in ~90% and ~20% reductions in carbonate excretion, respectively (Mekuchi, Hatta and Kaneko, 2010). This was accompanied by a change in Mg^{2+} content from ~20-30% in control seawater, to ~5% in low-Mg^{2+} seawater and ~40% in low-Ca^{2+} seawater (Mekuchi, Hatta and Kaneko, 2010). This reinforces that Ca^{2+}, as opposed to Mg^{2+}, is the primary target for, and determinant of, precipitation. The proteinaceous organic matrix that regulates calcification in the gut is the most likely candidate for governing Mg^{2+} incorporation into carbonates (Schauer *et al.*, 2016).

The concentration of Ca^{2+} and Mg^{2+} in the ocean is directly proportional to salinity (Kato, 1966; Hwang and Lin, 2013). As such, the relationship between Ca^{2+}, Mg^{2+} and carbonate production is likely to be represented by the relationship between salinity and carbonate production. However, in any areas of the ocean with external Ca^{2+} input, it is possible that this could drive an increase in carbonate production. Over geological time, the concentration of Ca^{2+} and Mg^{2+} in seawater has varied independently of salinity and each other (Hain *et al.*, 2015). It is therefore important to understand the relationship between seawater Ca^{2+} and Mg^{2+} concentration and

carbonate production to be able to understand global $CaCO_3$ production over this time period.

Sulphate

As already mentioned, sulphate (SO_4^{2-}) is the dominant divalent anion in seawater. While SO_4^{2-} is not directly involved in the calcification reaction, it could affect carbonate production via inhibition of $CaCO_3$ precipitation. Sulphate can co-precipitate with marine biogenic calcites to form calcium sulphate, $CaSO_4$, to ~1 mol%, and can inhibit $CaCO_3$ precipitation (Morse, Arvidson and Lüttge, 2007). The proportion of SO_4^{2-} absorbed and excreted by the kidney, compared to the intestinal fluid, may therefore affect carbonate production. It has been predicted that elevated seawater SO_4^{2-} may decrease carbonate production (Wilson, 2014).

Phosphate

Phosphorous is an important nutrient for growth of all organisms, that is generally in low concentration in seawater, particularly in low-nutrient, low-chlorophyll areas, where it has been implicated in limiting phytoplankton growth and nitrogen fixation (Mills *et al.*, 2004; Ridame *et al.*, 2014). The concentration of phosphorous, in the form of phosphate, in seawater is unlikely to affect carbonate production. However, many fish consume prey with bony skeletons made up of calcium phosphate ($Ca_3(PO_4)_2$). This mineral is likely to be dissolved in the acid stomach, providing additional Ca^{2+} and phosphate ions (PO_4^{3-}). The interactions of PO_4^{3-} with calcification in the gut environment have not been studied, but in seawater PO_4^{3-} adsorbs to carbonate surfaces, exhibiting complex interactions with the carbonate surface and strongly inhibiting carbonate precipitation (Morse, Arvidson and Lüttge, 2007). In addition, it is possible that in the alkaline gut environment, dissolved PO_4^{3-} released from food in the acidic stomach, re-precipitates with Ca^{2+}. It is therefore likely that the presence of PO_4^{3-} in the gut will affect carbonate production.

The effect of feeding on gut carbonate production

As well as its osmoregulatory purpose, intestinal $CaCO_3$ precipitation is also proposed as a method for calcium homeostasis (Wilson *et al.*, 2009). Excretion of excess calcium at the kidney increases the likelihood of nephrocalcinosis. As fish consume prey whole, consumption of vertebrates containing calcium phosphate skeletons, or invertebrates with calcium carbonate shells, can represent a concentrated source of

calcium to the fish. This source of calcium may dramatically increase the quantity of ingested calcium available for precipitation in the gut, when compared to unfed fish where ingested seawater, and perhaps uptake at the gills, are the only sources of calcium.

Ingested food travels through the teleost digestive system in the same way as ingested seawater, undergoing acidification in the stomach and alkalinisation in the intestine. Calcium-containing bones or shells are likely to be partially or fully dissolved in the acidic stomach, liberating Ca^{2+} ions. As the intestines of fed fish are also high in HCO_3^-, ~60% the concentration of starved fish (Wilson *et al.*, 1996), it is likely that a significant amount of this Ca^{2+} will be precipitated as $CaCO_3$.

To my knowledge, only a single study has investigated the effect of feeding on carbonate production (Newbatt, 2015). This study involved feeding European seabass a ration equal to 2.5% of their body mass per day, which contained 2.06% calcium content. Consumption of this diet led to a 7.5-fold increase in carbonate production compared to the estimated carbonate production by starved fish. This may underestimate calcium intake in fish consuming similar sized meals composed of other fish, which are ~3.2% Ca^{2+} (Cameron, 1985). It appears that carbonate production increases linearly with Ca^{2+} intake in the diet, and importantly excretion rates in fed fish can be 10-times higher than starved fish (Newbatt, 2015). Further elucidation of carbonate production by fish fed realistically sized, natural diets is key to refining our estimates of global carbonate production by fish.

The presence of food in the intestine will change the environment that carbonates crystallise in, which could alter their composition and structure. However, evidence suggests that carbonates excreted by fed fish are similar in composition to those excreted when fasted (Salter, Perry and Wilson, 2012; Salter *et al.*, 2017). Incorporation of carbonates into faeces, as opposed to excretion in distinct pellets, is likely to affect sinking rate and dissolution of the carbonates, as well as the likelihood of disturbance by other organisms. If faeces reduce the sinking rate of carbonates, they are likely to remain in surface waters longer, leading to a greater degree of dissolution and restoration of alkalinity to these waters. Conversely, incorporation of carbonates into faeces may protect them from physical contact with corrosive seawater, leading them to dissolve more slowly.

Production of gut carbonates by fed fish is an intriguing avenue of research that remains largely unexplored. In the natural environment, fish spend a significant portion of their lifespan in a fed state, so data collected from starved fish is of limited usefulness for ocean carbon cycle modelling. In addition to differences in the quantity of $CaCO_3$ produced by fed fish, other related effects remain unknown. If the altered intestinal environment in which carbonates are precipitated affects composition and structure of excreted carbonates, their dissolution rate may be affected. In addition, incorporation of precipitates into faeces is likely to affect their sinking and dissolution rates, with implications for both the organic and inorganic carbon cycle.

Pressure

An intriguing and understudied feature of the ocean with regards to fish gut carbonates is hydrostatic pressure. In the ocean, hydrostatic pressure increases by ~1 atmosphere (atm) for each 10 m increase in depth (Yancey *et al.*, 2014). Hydrostatic pressure has an inhibitory effect on cellular structures. Deep-sea organisms have been found to have adaptations that confer pressure resistance, with the osmolyte tri-methylamine *N*-oxide (TMAO) implicated in stabilising proteins against pressure up to ~8,500 m depth, which is the maximum depth at which teleosts have been observed.

As well as affecting cellular structures, pressure influences the gases dissolved in seawater, and the carbonate chemistry system. Calcium carbonate saturation state decreases with increasing pressure, and the depth at which $\Omega=1$ is known as the saturation horizon. The depth of this horizon varies with temperature and CO_2 concentration, because this affects the concentration of CO_3^{2-} in the seawater, although deep water is relatively stable with regards to temperature and CO_2 compared to surface water. As pressure increases with depth, carbonates become more likely to dissolve as they sink.

As previously mentioned, conditions inside the intestine of marine fish are extremely favourable to precipitation of $CaCO_3$ compared to seawater. It is therefore unlikely that pressure, even in the deepest areas of the ocean that fish inhabit, will decrease Ω-$CaCO_3$ enough to halt precipitation, or to inhibit precipitation significantly. Indeed, dissection of mesopelagic fish brought to the surface by trawlers has revealed the presence of carbonates in their guts, although it is possible that these could have precipitated as the fish were brought to the surface. However, it is more likely that

rapidly increased CO_2 in the intestine caused by rapid ascent would result in dissolution of some carbonates between being caught and dissection. Research on fish under high pressure is extremely challenging, but it would be interesting to investigate the effects of pressure on carbonate production, particularly with reference to the upward alkalinity pump.

Fishing

The size structure of fish populations is determined by a number of biotic and abiotic factors, and different sized fish contribute varied ecological roles. Fishing is a prominent driver changing the size structure of fish populations as in many fisheries, fishing selectively removes larger fish due to their greater economic value (Hixon, Johnson and Sogard, 2014). While this removes biomass, and therefore reduces carbonate production, if the difference in biomass is made up by more smaller fish this could actually result in an increase in carbonate production overall. Fishing is thought to have decreased the total biomass of fish communities, and to have shifted the size structure towards smaller individuals (Bianchi *et al.*, 2000; Shin *et al.*, 2005). Indeed, the biomass of predatory fish, which are generally targeted by fisheries, is thought to have declined by up to two thirds in the last century, accompanied by increases in prey fish (Christensen *et al.*, 2014). Predator removal has already led to declines in algal communities and habitat loss (Silliman *et al.*, 2005; Wilmers *et al.*, 2012).

Many fisheries are now managed in such a way as to produce maximum sustainable yield. This refers to the maximum amount of the population that can be removed per year, while maintaining the population size and maximising its growth rate. Modelling has revealed that rates of fishing lower than those associated with maximum sustainable yield result in substantial reductions in carbonate production by fish (Jennings and Wilson, 2009).

Marine reserves have been proposed as a measure to enhance the ability of the ocean to absorb CO_2, by rebuilding fish populations whose excretion of bioavailable nitrogen stimulates primary productivity (Roberts *et al.*, 2017). Due to dwindling stocks of traditionally fished species, there is interest in expanding fisheries to include previously undesirable fish, such as mesopelagics (St. John *et al.*, 2016). It may be important to protect species implicated in the 'upward alkalinity pump', with open ocean marine reserves being suggested as a method for this (Roberts *et al.*, 2017).

It is difficult to disentangle the influences of fishing from other stressors, such as temperature, on global fish biomass and the size structure of populations. However, it seems unlikely that increases in abundance of small fish will make up for reductions in overall biomass associated with fishing. Therefore, the impact of fishing is likely to be a reduction in total carbonate production by fish.

Conclusion

Many important gaps still exist in the literature, limiting our ability to make accurate estimates of the current contribution of fish to global $CaCO_3$ production, and how this will change in the future in relation to climate change. The effect of seawater CO_2 concentration is comparatively well studied compared to other factors affecting carbonate production. Elevated CO_2 appears to increase HCO_3^- secretion into the intestine, but this does not necessarily equate to an increase in carbonate excretion, suggesting that this process may be more limited by Ca^{2+} availability than HCO_3^- supply. All evidence suggests that elevated temperature and salinity increase carbonate excretion, in an exponential fashion. Overall, it appears likely that climate change will increase the contribution of fish to global marine carbonate production, but the effects on ocean chemistry are complicated by additional effects of CO_2, temperature, and other factors on the magnesium content of carbonates and their solubility in seawater.

I identify three areas of particular importance to future research on carbonate production by marine fish. Firstly, investigation of the effects of small changes in salinity on stenohaline marine species, as these represent a majority of marine species and may be much more sensitive to small changes in salinity than euryhaline species. Secondly, how carbonate excretion rates of fish are affected by temperatures <10 °C, as this temperature range is underrepresented in the literature, and many fish likely spend a significant portion of their lifespan residing in cold water. Thirdly, the effect of food consumption and diet on carbonate production, as almost all current estimates of global carbonate production are based on measurements from starved fish, which is not representative of the state of most marine fish worldwide at any given time.

Aims of the project

In this project, I aim to tackle two of the aforementioned important gaps in current knowledge of carbonate production by fish. Firstly, I have investigated the effect of low temperatures on gut carbonate excretion and excreted carbonate composition, using lumpfish, *Cyclopterus lumpus*, as a cold-tolerant study species. This will help to refine estimates of global carbonate production by fish and be the first study (to my knowledge) investigating carbonate production below 5 °C. Secondly, I have investigated the effect of diets containing environmentally relevant levels of calcium salts on the blood and intestinal chemistry, and carbonate products, produced by freshwater-acclimated rainbow trout, *Oncorhynchus mykiss*. Although the latter is not under marine conditions, a surprising observation from the experiments of another postgraduate student during my time at Exeter revealed that freshwater fish can also produce intestinal carbonates, if provided with diets with calcium levels resembling natural prey. This raised the intriguing possibility that the evolution of intestinal carbonate production in fish was not exclusive to the marine environment. This provided me with a unique opportunity to explore the relationship between feeding, intestinal chemistry, and carbonate production, in the absence of osmoregulation-related intestine functions related to inhabiting a hyperosmotic environment.

Chapter 2: The effect of temperature on the quantity and composition of excreted carbonates in lumpfish, *Cyclopterus lumpus*

Introduction

The marine inorganic carbon cycle involves the precipitation and dissolution of calcium carbonate ($CaCO_3$), and the balance of these two processes directly influences atmospheric CO_2 concentrations. Traditionally, marine calcifying microorganisms, such as coccolithophores and foraminifera, are thought to be quantitatively the most significant contributors to oceanic $CaCO_3$ production (Feely *et al.*, 2004), along with smaller contributions from coralline algae, echinoderms, and other invertebrates. However, it has been highlighted that fish may account for 3-45% of oceanic $CaCO_3$ production (Wilson *et al.*, 2009).

Understanding the contribution of fish to $CaCO_3$ production is important due to the large quantity and unique mineralogy of fish-derived carbonates, at least some of which appear to be more soluble than other biogenic $CaCO_3$ (Woosley, Millero and Grosell, 2012). This is due to the incorporation of magnesium carbonate ($MgCO_3$) with $CaCO_3$ to form high-magnesium calcite (Perry *et al.*, 2011; Salter, Perry and Wilson, 2012; Salter, Perry and Smith, 2019), of up to 54 mole% Mg^{2+} (Foran, Weiner and Fine, 2013), although pure $MgCO_3$ has been observed in the form of amorphous magnesium carbonate (AMC) (Salter *et al.*, 2018). Fish derived $CaCO_3$ is excreted throughout the lifetime of the fish as a by-product of osmoregulation, so the location of its precipitation and dissolution can be affected by fish behaviour (Wilson *et al.*, 2009). This is in contrast to microorganisms that build their exoskeletons from $CaCO_3$, whereby most $CaCO_3$ only becomes vulnerable to significant net dissolution upon death of the organism. This unique mineralogy and influence of fish behaviour could result in fish-derived carbonates having different effects on ocean chemistry compared to those produced by plankton and microorganisms (Wilson *et al.*, 2009; Wilson, 2014).

Sea surface temperature (SST) varies from just above the freezing point of sea water in polar areas, to over 30 °C in the tropics. As well as increasing CO_2 concentrations in the atmosphere and seawater, climate change is also increasing ocean temperatures, with 2019 being the ocean's warmest year on record at the time (Cheng

et al., 2020), and SST is expected to be 1-3 °C warmer by 2100 (IPCC Panel, 2014). As biological reactions tend to be temperature sensitive, knowledge of this sensitivity is important to understanding biological processes in different areas of the ocean, and how they may be affected by future climate change.

A temperature quotient (Q_{10}) is used to represent the temperature sensitivity of biological rate functions. Specifically, it refers to the effect of a 10 °C increase in temperature on rate functions, which can include whole-body measures such as metabolic rate. A Q_{10} of 1 would mean a reaction is not temperature sensitive, while a Q_{10} of 2 or 3 would mean that rate doubles or triples, respectively, for every 10 °C increase in temperature. While Q_{10} is widely used to describe the temperature sensitivity of rates as exponential, there is evidence that some species may not fit this assumption across their entire thermal range, and may be able to regulate standard metabolic rate to be relatively constant across the middle portion of their temperature tolerance range (Gräns *et al.*, 2014).

Marine teleost fish must drink seawater to avoid dehydration, due to inhabiting an environment that is hyperosmotic (~1000 mOsm/l) to their tissues (310 – 350 mOsm/l (Larsen *et al.*, 2014). Ingested seawater is desalinated in the oesophagus and enters the stomach at ~500 mOsm/l, due to absorption of Na^+ and Cl^- (Parmelee and Renfro, 1983; Nagashima and Ando, 1994). Desalinated seawater is then acidified in the stomach to a pH of ~2-4, resulting in the conversion of all carbonate (CO_3^{2-}) and bicarbonate (HCO_3^{2-}) to CO_2 (Pedersen, Colmer and Sand-Jensen, 2013). Desalinated and acidified seawater subsequently enters the anterior intestine, which is the major site of water absorption. Solute-coupled water absorption is facilitated by a combination of NaCl uptake and anion (Cl^-/HCO_3^-) exchange (Wilson, Wilson and Grosell, 2002; Grosell, 2019). Elevated intestinal HCO_3^- content resulting from intestinal anion exchange, combined with high concentrations of Ca^{2+} and Mg^{2+} from ingested seawater favour precipitation of $CaCO_3$ and $MgCO_3$ (hereby referred to as carbonates). In the absence of divalent ion precipitation, Ca^{2+} and Mg^{2+} would become even more concentrated and impede water absorption. The precipitation of carbonates further reduces intestinal osmolality by ~70 – 100 mOsm/l, to be approximately isosmotic to plasma (Grosell and Taylor, 2007). This indirectly facilitates water absorption by reducing the osmotic gradient to within the range within which water absorption is possible (up to 35 mOsm/l) (Genz, Esbaugh and Grosell, 2011).

Carbonates are then excreted as mucus-coated pellets or incorporated with faeces if the fish is feeding (Walsh *et al.*, 1991; Wilson *et al.*, 1996; Wilson, Wilson and Grosell, 2002).

Wilson et al. (2009) predicted the contribution of fish to global $CaCO_3$ production based on two species, European flounder (*Platichthys flesus*) and gulf toadfish (*Opsanus beta*), a temperate and subtropical/tropical species, respectively. These estimates were based on a carbonate production Q_{10} of 1.83 (Wilson *et al.*, 2009), which represents the mean Q_{10} for metabolic rate of 69 species over a range of 0-30 °C (Clarke and Johnston, 1999). However, carbonate production is likely to be more sensitive to temperature than metabolic rate, due to higher Q_{10} for temperature associated increases in drinking rate (Takei and Tsukada, 2001), which provides more Ca^{2+} (in the ingested sea water) for calcification in the intestine. Indeed, all tested species have a carbonate production Q_{10} higher than the average Q_{10} for metabolic rate (Reardon *et al.*, in preparation; Wilson *et al.*, 2009; Heuer *et al.*, 2016), with a carbonate production Q_{10} of 2.33 and 3.21 for sheepshead minnow (*Cyprinodon variegatus*) and gulf toadfish, respectively (Wilson *et al.*, 2009; Heuer *et al.*, 2016).

The aforementioned Q_{10}s were calculated from carbonate production rates measured at temperatures above 20 °C (Wilson *et al.*, 2009; Heuer *et al.*, 2016). These estimates can therefore be applied to subtropical and tropical areas of the ocean, which can be characterised by high fish biomass, such as coral reefs. However, many species live in temperate or polar areas of the ocean, where water rarely, if ever, reaches 20 °C. In addition, species including mesopelagics, which may account for >90% of global fish biomass (Irigoien *et al.*, 2014), spend much of their time in or below the thermocline, so are regularly exposed to temperatures as low as 4 °C. An understanding of how carbonate production is affected at low temperatures is therefore essential to refining our understanding of $CaCO_3$ production on a global scale. In addition to the effects of temperature on carbonate production, the saturation state (Ω) of carbonates in seawater is positively correlated with temperature, thus carbonates will dissolve more quickly at lower temperatures.

Lumpfish (*Cyclopterus lumpus*) inhabit a wide geographic range, from the Arctic in the north to temperate regions in the south, 70° to 37°N respectively (Nytrø *et al.*, 2014; Treasurer, 2018). The species is generally found in waters 50 to 150 m deep, but has

been recorded at depths up to 868 m (Parin, Fedorov and Sheiko, 2002; Treasurer, 2018). As the August (warmest month) surface water temperatures in areas the lumpfish inhabit can range from 0 - 20 °C, lumpfish can be seen to tolerate a wide range of temperatures, including tolerance to very cold temperatures (Treasurer, 2018). We therefore chose lumpfish as a suitable species to investigate how cold temperatures affect the production of $CaCO_3$ precipitates. It is also more reliably available than wild-caught species due to their production as a cleanerfish in the salmon aquaculture industry.

Significant mortality and erratic behaviour of lumpfish has been observed at 18 °C, as well as cataract formation at 15 °C (Hvas *et al.*, 2018). This suggests that welfare of lumpfish may be compromised at or even below 15 °C. As we were primarily interested in the effect of cold temperatures on $CaCO_3$ precipitate production, we decided to investigate the effects on this within a temperature range of ~2 to ~12 °C.

Materials & methods

Animals and experimental conditions

Lumpfish (n= 19; body mass: 168.7 ± 7.9 g) were obtained from Ocean Matters Ltd. Anglesey. Upon arrival (Aquatic Resources Centre, University of Exeter), fish were stored in a ~500 L stock tank provided with fully aerated artificial seawater (35 ppt) in a miniature recirculating system, made up with dechlorinated tap water and commercial sea salt (Tropic Marin), at ~13 °C. Food was provided daily, equalling a total of 2% of the total biomass of fish in the tank, divided between a morning and afternoon feed. Nineteen fish were then placed in 19 tanks, with each set of 5 (or 4) tanks fed by a separate water recirculation system within a temperature-controlled cabinet at a nominal temperature of 12 °C. Each individual tank, as well as the sump tank, were provided with aeration. A mesh was installed over the outflow of the tank to prevent any buoyant precipitates, or precipitates that were lifted by air bubbles, from being removed from the tank by water flow. Air stones were positioned slightly above the tank floor to reduce interaction between carbonates and air bubbles.

As seawater is more corrosive to $CaCO_3$ at lower temperatures, we artificially manipulated seawater alkalinity to minimise dissolution of precipitates between excretion and collection. When marine fish drink, seawater is acidified in the stomach and all HCO_3^- and CO_3^{2-} is converted to CO_2. We were therefore confident that enhanced seawater alkalinity would not affect carbonate production by altering intestinal fluid alkalinity. Using CO2Sys v2.3 and the published stoichiometric solubility constant (pK^*sp) for gut carbonates produced by gulf toadfish, which is 1.95 times that of aragonite (Woosley, Millero and Grosell, 2012), we calculated that an alkalinity of 3000 µmol/kg is sufficient to keep the saturation state (Ω) for fish carbonates above 1 at all temperatures (K1, K2 from Lueker *et al.*, 2000, $KHSO_4$ from Dickson, 1990, KHF from Perez and Fraga, 1987 – these constants were used throughout this chapter whenever CO2Sys was utilised), minimising dissolution. Sodium bicarbonate ($NaHCO_3$) was added to the artificial seawater to elevate alkalinity to 3000 µmol/kg, and this was monitored and topped up weekly.

Prior to carbonate collection, fish were fed a ration of 2% body mass, and any feed not consumed was noted before removal after ~12 hours. Fish were then left for at least 60 hours, in order to allow for complete processing and excretion following the meal, so that excreted carbonates were not accompanied by products of digestion. After

these 60 hours had passed, all faeces and carbonates were removed from the tank and disposed of. Following this, carbonates were collected from the tank floor and outflow mesh into sample tubes twice per day for 72 hours. Carbonates were collected from the tank floor and outflow mesh into sample tubes twice per day for three days, in order to minimise interaction of carbonates with seawater, consistent with other studies (Salter, Perry and Smith, 2019). Fish species that are large or inactive have previously been observed to have stochastic carbonate excretion rates at 10 °C (Salter, Perry and Smith, 2019). Additionally, we observed that there was inter-individual variability in carbonate excretion rates, including some fish that appeared to excrete little or no carbonate. In order to minimise the effect of low temperature-associated stochasticity, as well as inter-individual variability on our results, we decided to expose the same fish to all three temperatures. All fish were sequentially exposed to nominal temperatures of 12, 7 and then 2 °C (n = 19 for all temperatures). This was achieved by reducing the set point on the temperature-controlled cabinet to the desired temperature. A stable temperature was reached within 24 hours of the set point being adjusted, although in the case of the 2 and 12 °C exposures, the temperatures achieved were ~1-2 °C higher than the nominal temperature.

Water chemistry analysis

At the end of each carbonate collection period 12 ml water samples were taken and poisoned with 38 µl of 147 mM $HgCl_2$, according to Dickson *et al*., (2007), to allow for measurement of water total CO_2 (TCO_2). In addition, pH, salinity, and temperature were measured using (Mettler-Toledo SevenCompact Duo S213), to allow for calculation of carbonate chemistry parameters. Seawater DIC analysis was conducted using a custom-built system based on that of Friedrich et al. (2002), as described in Lewis (2013). In essence, precisely 1 ml of seawater is added at a rate of 20 µl per second to a chamber containing 0.05 ml 0.2% phosphoric acid solution. A constant flow of CO_2-free N_2 through the chamber carries CO_2 which has been off gassed from the water through a cylinder of magnesium perchlorate (to remove all water vapour) into a non-dispersive infrared analyser (Qubit S154 IRGA CO_2 analyser). Outputs of data were collected and analysed using LabProTM Interface and Loggerpro v3.2. From this, carbonate chemistry parameters were calculated using CO2Sys_v2.3.

Precipitate analysis

Following carbonate collection, excess seawater was removed, and precipitates were briefly rinsed in deionised water to remove extraneous sea salts. Deionised water was then replaced with a 4.5% sodium hypochlorite (NaClO) solution, equal to at least ten times the mass of the carbonates. After >48 hours in NaClO, all precipitate samples from a given fish at a single temperature were combined into a single tube for analysis. Excess sodium hypochlorite was removed, and precipitates were rinsed three times with deionised water. Excess deionised water was removed, then precipitates were dried at 50 °C overnight, or until carbonates were completely dry.

Prior to preparation for titration, the mass of the tubes containing compiled dry precipitates were measured to allow calculation of dry mass. A small subsample for SEM-EDX was then removed from each of the five largest carbonate samples per temperature, equal to <1% of the total mass of carbonate produced by this fish. This was accounted for by increasing the final titrated carbonate concentration by the proportion of dry mass that was removed. Precipitate mass was then estimated by eye to allow allocation of each to an appropriate titrant strength in order to measure bicarbonate equivalent (HCO_3^- & CO_3^{2-}) content accurately. Precipitates were prepared for titration by addition of 2.5 ml ultrapure water, followed by sonication, then added to a titration vial. This was repeated 8 times to ensure removal of all precipitates from the tubes, and to reach a final volume of 20 ml. After all precipitates were removed from the tubes, the mass of the tubes was measured and subtracted from the mass of the tubes containing precipitates in order to calculate dry mass of the precipitates. For estimation of carbonate production rate based on mass of excreted carbonates, the dry mass of the precipitates after processing were assumed to be 100% $CaCO_3$.

Bicarbonate equivalent content of precipitates was ascertained by double endpoint titration using an autotitrator (Metrohm 870 Dosino dosing units, 815 Robotic USB sample processor (XL), 970 Titrando controlled by Tiamo titration software (v2.3)). Under constant bubbling with nitrogen, initial pH of the solution was measured. Next, acid was added to the sample until it reached pH 3.89, at which pH all HCO_3^- and CO_3^{2-} had been converted to CO_2, which was gassed off by the nitrogen. Once the pH stabilised, indicating all HCO_3^-, CO_3^{2-} and CO_2 had been removed from the sample, the amount of acid added was recorded. Equimolar base was then added to the

solution until pH returned to the initial measured value. The amount of CO_3^{2-} in the sample was then calculated as follows:

$$CO_3^{2-} = \frac{Acid - Base}{2}$$

Where *[CO₃²⁻]* refers to the amount of CO_3^{2-} in the precipitate in µmoles, *Acid* refers to µmoles of acid added to reach pH 3.89 and *Base* refers to µmoles of base added to return the sample to the initial measured pH. Because CO_3^{2-} is a divalent ion, it requires 2 H^+ ions to titrate. Calcium carbonate excretion rate was then calculated as follows:

$$R = \frac{[CO_3^{2-}]}{W_F \times t}$$

Where *R* refers to excretion rate of carbonate precipitates (including $MgCO_3$ and $CaCO_3$ – measured either directly by titration, or calculated from dry mass assuming precipitates were 100% $CaCO_3$) in µmol/kg/h, *W_F* refers to the mass of the fish in kg, and *t* refers to the period over which precipitates were excreted and collected, in hours. The change in rate of carbonate excretion between acclimation temperatures was expressed as a Q_{10}, calculated as follows:

$$Q_{10} = (R_2 - R_1)^{10/(T_2 - T_1)}$$

Where *R_1* and *R_2* were rates of carbonate excretion calculated at different temperatures, with *R_2* being the carbonate excretion rate at the higher temperature, and *T* refers to the corresponding temperatures at which each carbonate excretion rate was calculated.

Scanning electron microscopy, energy dispersive x-ray spectroscopy (SEM-EDX) was used to visualise carbonates, and to assess magnesium content of precipitates. Subsamples of precipitate produced at each temperature were mounted on carbon tape and coated with a 10 nm gold/ palladium coating. Fish carbonates with ellipsoid morphology were visually identified using SEM, and three points per pellet, and five pellets per fish, from five fish from each treatment, were analysed with EDS to obtain calcium and magnesium content. $MgCO_3$ mol% was calculated as follows:

$$MgCO_3 \; mol\% = \frac{MgA}{MgA + CaA} \times 100$$

Where *MgA* and *CaA* refer to the Mg and Ca atomic % as measured by EDX, respectively.

Further precipitates from 12 °C were analysed by attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy to identify carbonate polymorph. Using a Nicolet 380 FTIR spectrometer coupled with a Thermo Scientific SMART iTR ATR sampler equipped with a diamond reflecting cell, scans were performed at a resolution of 2 cm^{-1}, and final spectra were obtained via co-addition of 32 repeated scans (Salter, Perry and Smith, 2019). Spectra were then plotted as infrared absorbance vs wavelength, with peak positions, intensities, and shapes as a function of the molecular vibrations within the sample. Absorbance peaks generated by the four vibrational modes of inorganic carbonates are polymorph-specific, therefore allowing identification of carbonate polymorphs, via reference to a spectral database (Salter *et al.*, 2017; Salter, Perry and Smith, 2019).

Statistics

All statistical analyses were performed in Graphpad Prism 9. In order to test the effects of acclimation temperature on carbonate production rate and $MgCO_3$ content, Shapiro-Wilk tests were first conducted to ascertain whether data were normally distributed. As data were not normal, non-parametric Friedman tests and Dunn's multiple comparisons tests were then used to calculate significant differences between carbonate production rates and $MgCO_3$ content at different temperatures.

Results

Mortality and behaviour observations

No mortalities occurred at any temperature during the data collection period of this study. One mortality occurred during the 8-week isolated acclimation period at 12 °C, prior to the start of the experiment. Although this mortality occurred at the highest temperature treatment, this was the temperature to which the fish were exposed to for longest. We therefore did not attribute this mortality as an effect of temperature.

All fish were observed to feed at all temperatures, although food consumption appeared to be reduced at lower temperatures. Fish were significantly more active at the 12 °C treatment, spending more time swimming, and less time attached to the surfaces of the tank. Some individuals at 12 °C were also observed swimming at the surface of the water, often with their heads protruding above the water surface, as has been observed in lumpfish at 18 °C (Hvas *et al.*, 2018). While not quantified, these observations have relevance for interpretation of the results of this experiment.

Water chemistry

Table 1: The seawater physical and carbonate chemistry parameters associated with each treatment. Data are represented as means ± standard error. The saturation state (Ω) of fish-produced magnesium-calcite is calculated based on the assumption that it is 1.95 times more soluble than aragonite.

Nominal temperature (°C)	Measured temperature (°C)	pH_{NBS}	Salinity (ppt)	TCO_2 (µmol/kg)	TA (µmol/kg)	pCO_2 (µatm)	HCO_3^- (µmol/kg)	CO_3^{2-} (µmol/kg)	ΩCalcite	ΩAragonite	ΩFish-produced magnesium-calcite
12	13.55 (±0.64)	8.06 (±0.01)	34.99 (±0.09)	2452.2 (±12.1)	2628.5 (±13.1)	593.7 (±5.2)	2289.0 (±11.7)	139.6 (±2.5)	3.32 (±0.06)	2.13 (±0.04)	1.09 (±0.02)
7	7.29 (±0.10)	8.00 (±0.04)	35.50 (±0.12)	2563.0 (±12.4)	2689.6 (±18.1)	673.1 (±68.0)	2420.9 (±15.1)	109.9 (±10.1)	2.61 (±0.24)	1.66 (±0.15)	0.85 (±0.07
2	3.33 (±0.25)	8.10 (±0.01)	35.31 (±0.17)	3108.2 (±46.8)	3280.1 (±42.8)	616.2 (±19.3)	2926.6 (±47.3	149.0 (±3.04)	3.55 (±0.07)	2.24 (±0.05)	1.15 (±0.02)

Carbonate production rate

Two fish appeared not to excrete any carbonates at any temperature, although as fish were not physically separated from carbonates, it is possible that they were eaten. The number of fish that excreted no carbonates throughout the whole 3-day sampling period increased at lower temperatures, with 0, 2, and 7 of the remaining 17 fish excreting no carbonates at the 12 °C, 7 °C and 2 °C treatments, respectively. The two fish that produced no carbonate at 7 °C did produce carbonate at the 2 °C, while one fish excreted approximately double the amount of carbonate at the 2 °C compared to the 7 °C.

The carbonate excretion rate of lumpfish increased with acclimation temperature. There was no significant difference between carbonate excretion rates at 2 °C and 7 °C, as measured by titration (P=0.1547) or mass (P=0.4329). There was a significant difference between carbonate excretion rates at 2 °C and 12 °C measured by titration and mass (both P=<0.0001), and a significant difference between carbonate excretion rates at 7 °C and 12 °C measured by both titration (P=0.002) and mass (P=0.0003). The Q_{10} was 3.50 when calculated between 2 °C and 7 °C as measured by titration and was 2.67 when calculated based on carbonate mass. A larger Q_{10} of 9.04 for carbonate excretion rate was observed between 7 °C and 12 °C as measured by titration, or 11.30 as measured by mass. The overall carbonate excretion rate from 2 °C to 12 °C was 6.25, as measured by titration, or 6.46 as measured by mass. Measures of carbonate production by mass were consistently ~2.3-fold higher (standard error = 0.1) than when measured by titration across all treatments, although this difference was slightly reduced at 7 °C, where production as measured by mass was ~2.1-fold higher.
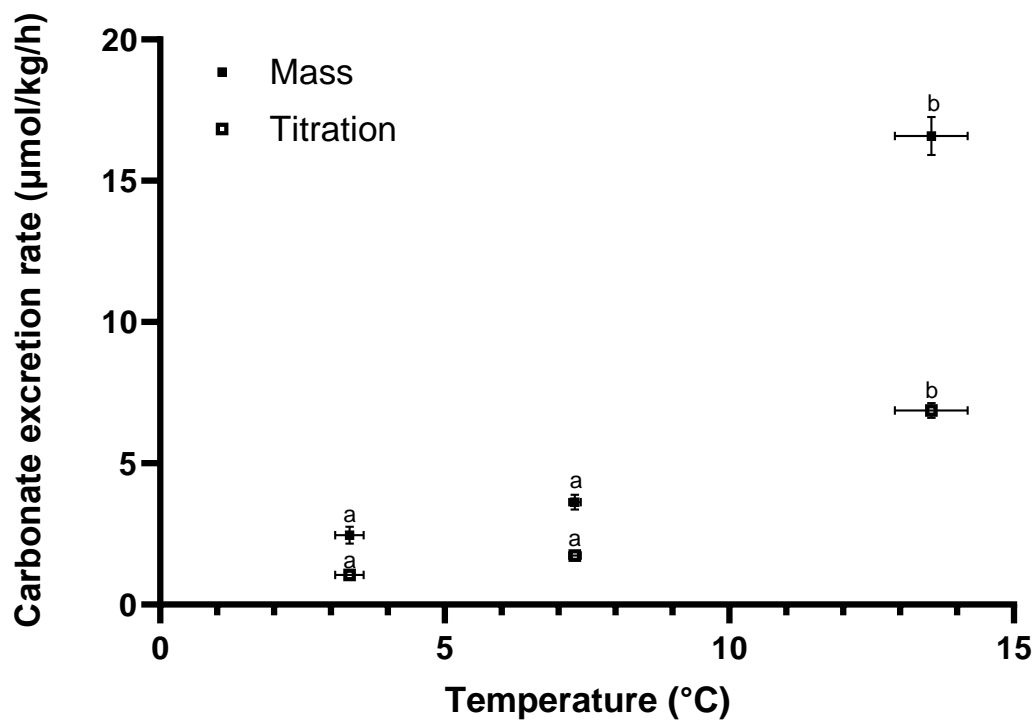
Figure 3: Carbonate excretion rate by lumpfish. Filled squares represent carbonate excretion rate calculated by weighing dry mass of excreted carbonates, then calculating molar equivalent assuming excreted carbonates were 100% $CaCO_3$. Unfilled squares represent carbonate excretion rate calculated by titration of all CO_3^{2-} within excreted precipitates. Statistically significant differences are indicated by different letters. N=19. Data are mean ± standard error. Some error bars are not shown as they are small enough to be obscured by data points.

Magnesium content of precipitates

Acclimation temperature did not affect magnesium content of excreted precipitates (P=0.0718).
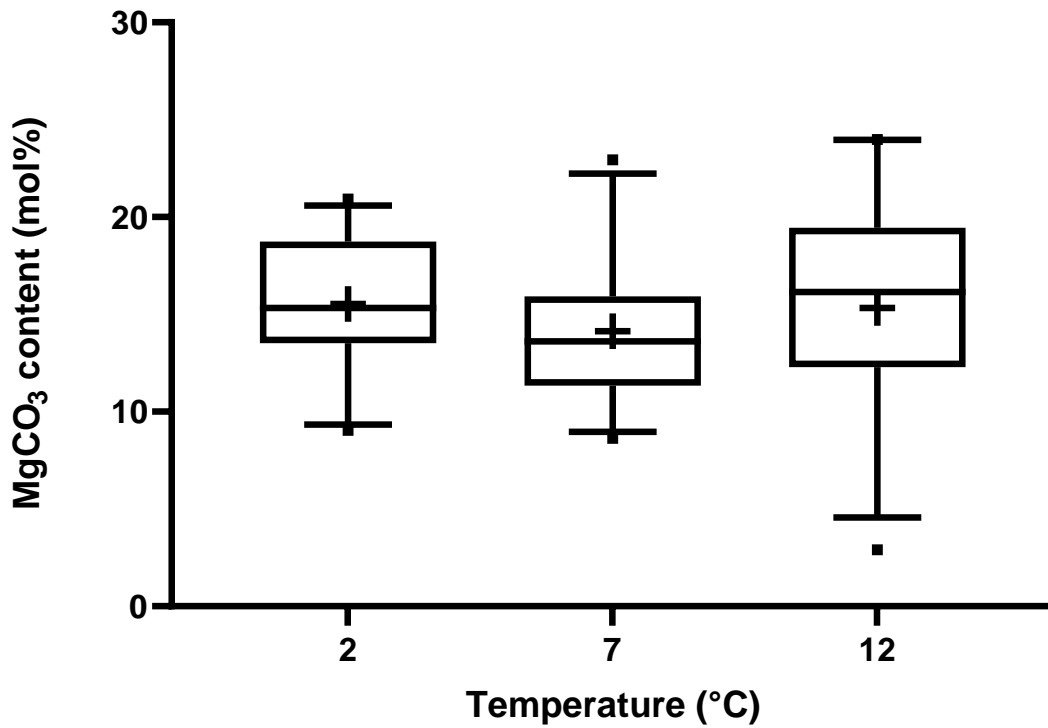
Figure 4: Magnesium content of carbonate precipitates excreted by lumpfish. Box represents 25-75 percentile, while whiskers represent 5-95 percentile. The midline of the box represents median, while the cross represents mean. Filled squares represent outliers. There are no statistically significant differences between treatments.

Mineralogy and morphology of precipitates

Carbonate peaks suggest partially crystallised calcite. There is also a small peak indicating brucite ($\sim$3500 cm^{-1}) and a broad O-H^{-} stretch peak (where vibrations are related to the stretch between these atoms), suggesting the presence of water in the structure of the crystals. The morphology of carbonates was typically ellipsoids in the 2-4 µm range.
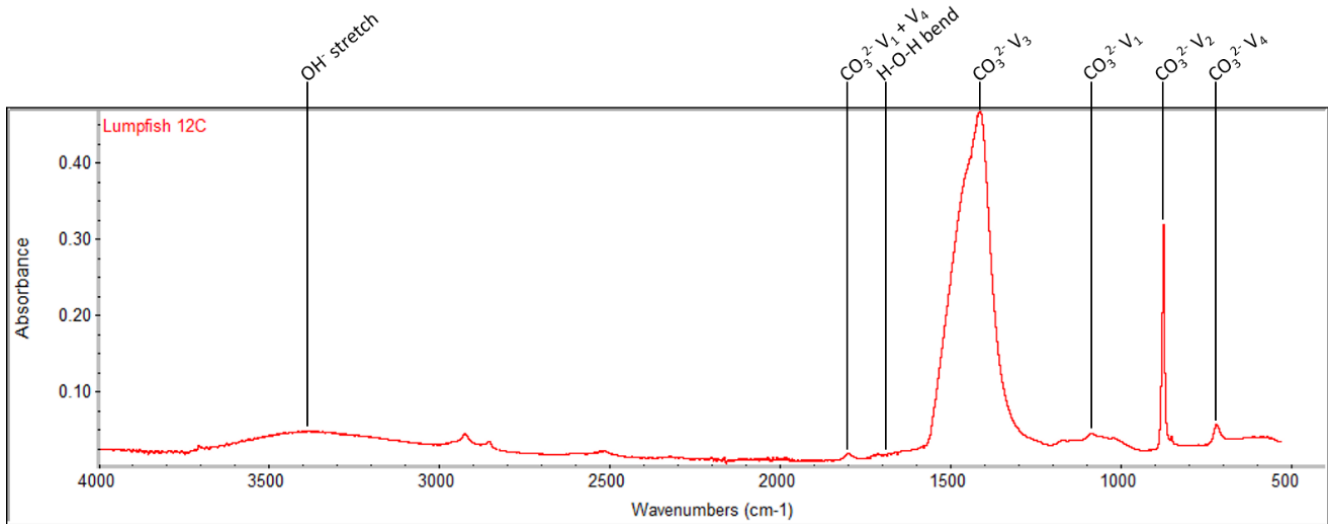
Figure 5: Representative ATR-FTIR spectrum for the carbonate polymorph produced by lumpfish at 12 °C.
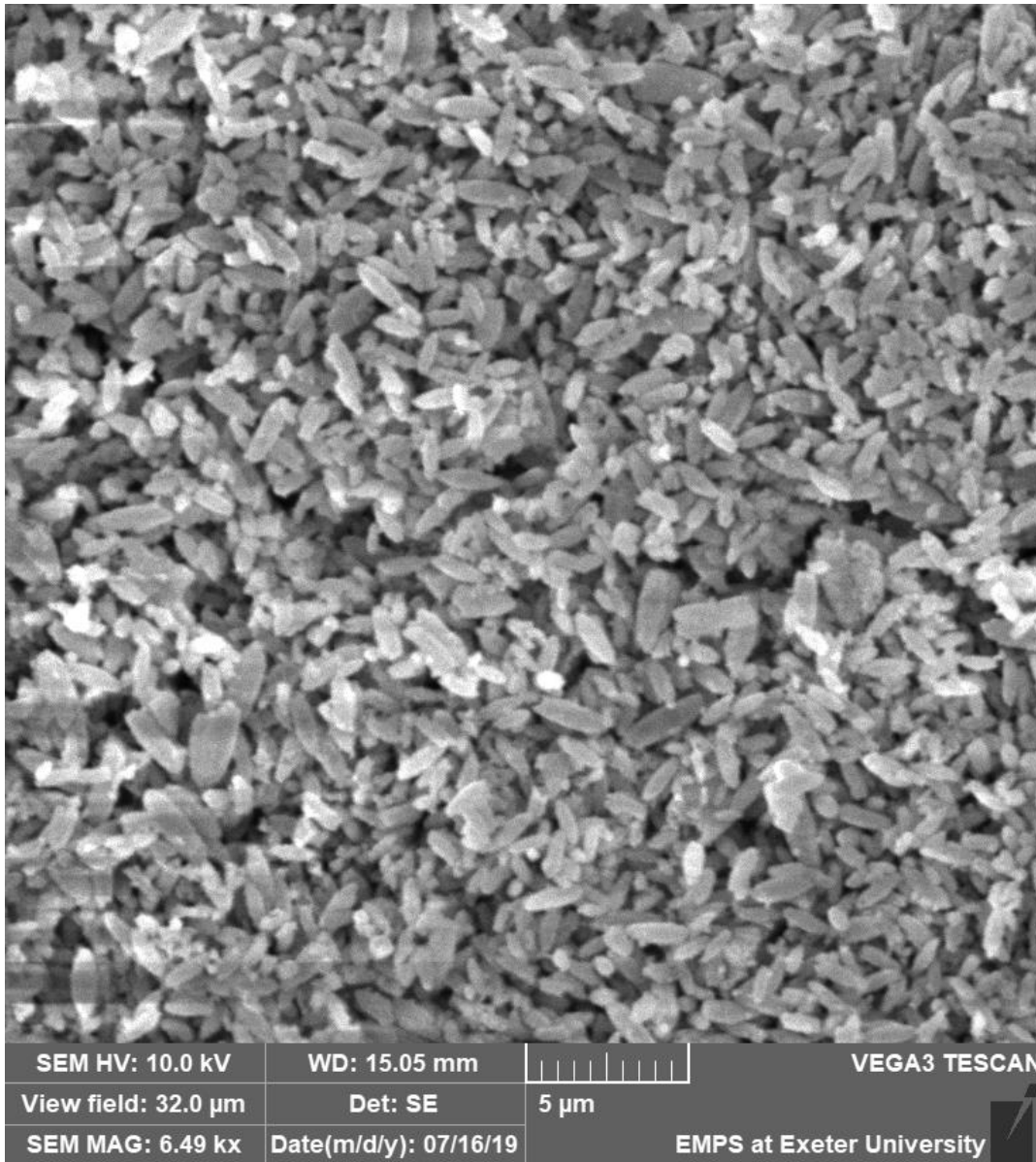
Figure 6: Scanning electron micrograph of carbonate precipitates produced by lumpfish at 12 °C, which have been bleached and coated with 10 nm Au/ Pd.

Discussion

The discrepancy between the values for $CaCO_3$ measured by titration and dry mass (assuming 100 % $CaCO_3$) is puzzling. Dry mass gave a value ~2.3-fold higher than when measured by titration, with very little difference in ratio based on temperature. Incorporation of $MgCO_3$ into precipitates cannot explain this difference, as magnesium has a lower molar mass than calcium, 24.3 g/mol compared to 40.0 g/mol, so presence of $MgCO_3$ would lead us to underestimate CO_3^{2-} content based on dry mass. In contrast, incorporation of calcium phosphate $Ca_3(PO_4)_2$ into precipitates could explain the difference between the two calculated figures. As $Ca_3(PO_4)_2$ has a much greater molar mass than $CaCO_3$, 310 g/mol as opposed to 100 g/mol, if the precipitates were ~28% $Ca_3(PO_4)_2$, this would explain the discrepancy in mass we found. However, while phosphate has been detected in carbonate samples (Salter, Perry and Wilson, 2012; Salter, Perry and Smith, 2019), it has not been detected at a high enough concentration to explain the differences in mass we detected. Additionally, FTIR analysis lacked a conspicuous phosphate peak that would be associated with high concentrations of $Ca_3(PO_4)_2$. The hydration peaks observed in FTIR spectra suggest that there was structural water associated with carbonates, which would also add mass to samples that would not be represented by CO_3^{2-} content. Calcination (heating to ~400 °C) can remove all structural water, and anecdotal evidence suggests that this can reduce mass of carbonates by ~30% (Salter, personal communication).

Methodological issues could also explain a part of the observed differences in carbonate mass. Part of the difference between the two methodologies may be explained by inclusion of materials that did not originate in the gut in collected carbonate samples. For example, fish scales and pieces of plastic from the tank or outflow mesh are possible candidates. In addition, if sonication did not effectively break up and disperse $CaCO_3$ pellets, clumps of $CaCO_3$ may not be dissolved fast enough for them to be measured in the acid titration, leading to an underestimation of carbonate content. It is also possible that electrostatic charge affected the calculation of dry mass as, if electrostatic charge was allowed to accumulate on the surface of plastic sample tubes more during the weighing of carbonate-containing tubes than empty tubes, this could lead to an overestimate of precipitate mass. There is evidence that electrostatic charge did influence some measurements, as some of the smaller samples returned a negative dry

mass (these were treated as having no mass in the analysis). This would suggest an underestimation of dry mass but demonstrates that accumulation of electrostatic charge can affect mass measurements. This may serve to explain why some dry mass measurements differed greatly from the visually estimated mass. For the above reasons, we will only refer to carbonate production rates as calculated by titration for the rest of this discussion. As the results were similar regardless of whether carbonate excretion was measured by mass or titration, the same conclusions would apply regardless of which was used.

Increased stochasticity of carbonate excretion at lower temperatures, indicated by the increased number of fish that did not produce carbonates throughout the whole sampling period, is consistent with other relatively inactive, bottom-dwelling fish (Salter, Perry and Smith, 2019). Our observations of reduced activity below 12 °C may have contributed to this stochasticity. Increasing the length of collection periods at lower temperatures, potentially up to 7 days, may help to reduce the effect of this stochasticity on results and produce a more accurate estimate of production rate per fish. Alternatively, use of more active, perhaps demersal, or pelagic, fish species may result in more regular excretion of carbonates and preclude the need for extended collection periods.

Calculation of a Q_{10} was complicated by rates of excretion at 7 °C being closer to those at 2 °C than those at 12 °C, which would not be expected if there was a consistent Q_{10} across the full range of tested temperatures. This could be because fish were allowed to acclimate to 7 °C for only ~60 hours prior to initiation of carbonate collection, whereas acclimation to 2 and 12 °C was for >240 hours. This increased acclimation time was due to a large observed increase in gut transit time at 2 °C, and the need to be sure that no food remained in the gut during experiments, which has also been observed in other species at low temperatures (Miegel *et al.*, 2010). However, multiple species have shown no evidence of changes in carbonate production throughout a sampling period that occurred following a 3 °C decrease in temperature, when collection started 48 hours after transfer (Salter, Perry and Smith, 2019). Additionally, previous studies have acclimated fish to temperatures for a similar period of 2.5-3.5 days (Heuer *et al.*, 2016). It therefore seems unlikely that these results were due to insufficient time to acutely acclimate to the new temperature.

Dissolution of carbonates between excretion and collection is another factor that may have contributed to differences in carbonate excretion rate between treatments. Because carbonates are more soluble in seawater at lower temperatures, we attempted to control seawater chemistry to minimise dissolution of carbonates prior to collection, but despite this endeavour alkalinity, and therefore saturation state, were lower at 7 °C than at 2 °C or 12 °C. This may have led to a greater degree of dissolution of carbonates at 7 °C than the other two temperatures, which could help to explain why carbonate production at 7 °C was lower than would be expected from an exponential relationship between 2 °C and 12 °C. However, as lumpfish carbonates exhibited lower magnesium content than those of gulf toadfish, ~15 mol% compared to ~48 mol% (Woosley, Millero and Grosell, 2012), and magnesium content of $CaCO_3$ is positively correlated with solubility (Morse and Mackenzie, 1990; Purgstaller *et al.*, 2019), lumpfish carbonates may be more stable and their Ω may have remained above or close to 1, even when alkalinity was lowest at 7 °C. Amorphous calcium and magnesium-rich carbonate (ACMC) is produced by a number of species in addition to other carbonate polymorphs (Salter *et al.*, 2017), but as amorphous calcium carbonate (ACC) solubility is around two orders of magnitude higher than that of calcite (Purgstaller *et al.*, 2019), any ACMC is likely to have dissolved quickly regardless of treatment, especially as incorporation of $MgCO_3$ into ACC increases its solubility even further (Purgstaller *et al.*, 2019). We did not find evidence of ACMC in our FTIR analysis of carbonates produced at 12°C, suggesting that either no ACMC was produced, or any ACMC that was produced was likely rapidly dissolved regardless of temperature. We therefore believe that the differences in alkalinity between treatments likely had little or no effect on the observed relationship of carbonate excretion rate with temperature.

Lumpfish standard metabolic rate (SMR) Q_{10} is higher between 9 and 15 °C than 3 and 15 °C (Hvas *et al.*, 2018), which likely contributes to the disparity between the differences in Q_{10} for carbonate excretion between 2-7 °C, and 7-12 °C, as carbonate excretion is tightly linked to metabolic rate (Wilson *et al.*, 2009). We anecdotally observed an increase in activity of lumpfish at 12 °C compared to 7 °C, which is consistent with hyperactivity that has been observed at 15 °C, but not 9 °C (Hvas, personal communication). The unique ability of lumpfish to attach to surfaces may help to explain the differences in Q_{10} calculated for 2-7 °C and

7-12 °C, as this behaviour may allow for energy saving (Hvas *et al.*, 2018). This elevated activity, or decrease in attachment time, likely caused a more dramatic increase in routine metabolic rate (RMR) between 7 °C and 12 °C than would be expected from the elevation in SMR alone, compared to the difference between 2 °C and 7 °C. While aforementioned factors of acclimation time and seawater alkalinity may have contributed to the discrepancy in calculated Q_{10}s, we believe that the possibility of a behaviour-driven elevation of RMR at 12 °C likely accounted for the majority of the difference between them.

This link between temperature, behaviour and carbonate excretion in lumpfish is interesting, but most other species lack the ability of lumpfish to attach to surfaces. It is true that most species reduce activity as temperature lowers, but it seems likely that this energy-saving attachment behaviour may affect RMR more than activity reductions in most other species. Therefore, when applying these results to other cold-water species, the Q_{10} for carbonate production from 2 °C to 7 °C is likely to be more applicable due to the greater consistency in lumpfish behaviour in these two treatments. This Q_{10} of 3.50 is also more consistent with research on other species (Reardon *et al.*, in preparation; Wilson *et al.*, 2009; Heuer *et al.*, 2016). Our observations, in combination with those of others (Hvas *et al.*, 2018), suggest that there may be a transition point in lumpfish behaviour within their natural temperature range, seemingly around 9-13 °C.

The anecdotally observed behavioural impacts of temperature may be exaggerated in fish kept in experimental conditions i.e., kept in fish tanks and provisioned with food. In their natural environment, lumpfish are likely to remain more active at lower temperatures due to the need for behaviours such as foraging. Lumpfish are known to continue foraging behaviour at low temperatures, making them a preferable cleaner fish species to wrasse, which undergo a period of winter dormancy (Kelly *et al.*, 2014; Powell *et al.*, 2018). We used the carbonate excretion rate data from 2 °C and 7 °C to calculate the Q_{10} because of the consistency in behaviour at these temperatures, but this may underestimate absolute values of carbonate excretion in the wild due to extensive usage of energy-saving attachment behaviour under experimental conditions. The Q_{10} calculated from these two temperatures probably represents the relationship between temperature and carbonate excretion well, but using this Q_{10}, along with the absolute value for carbonate production at 12 °C, may provide

a more realistic carbonate excretion rate at lower temperatures. Measures of RMR on lumpfish in the wild would be useful to help understand how carbonate production rate may vary between fish in experimental conditions compared to their natural environment.

Lumpfish appear to produce a form of weakly-crystalline calcite, similar to many temperate species (Salter, Perry and Smith, 2019). The lack of difference in magnesium content of lumpfish carbonates across temperatures is consistent with other data showing that most fish species' carbonate products are unaffected by temperature from 10-27 °C (Salter, Perry and Smith, 2019). This is in contrast to carbonates precipitated pseudo-homogenously from seawater, or biogenic calcites produced by organisms such as coralline red algae, which exhibit a positive correlation between temperature and $MgCO_3$ incorporation into $CaCO_3$ (Morse, Wang and Tsio, 1997; Halfar *et al.*, 2000).

The present study is the first to investigate how temperatures below 10 °C influence carbonate excretion by marine fish, which are likely to be broadly relevant to many temperate, polar, and deep-water species. The impacts of the warming ocean on fish species are complicated by range shifts to match temperature tolerance, along with declining biodiversity around the equator (Chaudhary *et al.*, 2021), which could lead to cold water species being pushed towards the poles. Polar species that are unable to shift their distribution to match their temperature tolerance will be forced to either tolerate warmer temperatures or become extinct. However, if fish biomass in a given area remains equal after warming, the elevated temperature should result in an increase in carbonate production, regardless of range shifts. So, if fish biomass remains similar under ocean warming, elevated temperature is likely to drive an increase in total carbonate production in the ocean by fish.

While many pelagic species may be able to shift their distributions to match their temperature tolerance, demersal, benthic and bentho-pelagic species such as lumpfish may be more constrained by availability of suitable habitat at appropriate depths (Rutterford *et al.*, 2015). This may be relevant to lumpfish as the hatchlings and juveniles may be obligate inhabitants of seaweed, where they live and feed (Vandendriessche *et al.*, 2007; Treasurer, 2018). If populations of lumpfish that live at the southerly end of their geographic extent are constrained from dispersing in such a way, ocean warming (Hvas *et al.*, 2018) may apply to

lumpfish in the wild. It is unclear from these short-term studies whether behavioural acclimation may occur over the long term associated with climate change.

In a warming ocean, carbonate Ω is higher, so carbonates are likely to dissolve more slowly and at greater depth. As precipitation at the surface, followed by dissolution at depth removes alkalinity from the surface ocean (Wilson *et al.*, 2009), the effect of ocean warming on carbonate solubility could reduce the ability of the surface ocean to absorb CO_2 from the atmosphere, acting as a positive feedback to climate change. This is in addition to the reduction in the solubility of CO_2 in the surface ocean due to warming, which reduces the ability of the ocean to absorb atmospheric CO_2 via the solubility pump (Cox *et al.*, 2000; Yamamoto, Abe-Ouchi and Yamanaka, 2018). Our data, and that of others, suggest that temperature has little or no effect on magnesium content, and therefore solubility, of carbonates produced by fish (Reardon *et al.*, in preparation; Salter, Perry and Smith, 2019), so temperature-associated changes in Ω of carbonates in seawater are likely to be the main driver of changes in dissolution under ocean warming.

The effect of small increases in temperature on carbonate excretion rate may be more significant than any small change in solubility, caused by magnesium content or the direct effect of temperature on carbonate dissolution. It is possible that temperature-driven elevations in carbonate excretion will result in a greater enough quantity of carbonates being produced that, even with reduced dissolution rates of individual carbonates, the net amount of carbonate dissolved in surface waters increases. The surface of the ocean is expected to warm by ~0.6-2 °C by the end of the 21st century (IPCC Panel, 2014). Assuming our Q_{10} of 3.50 is representative of all cold-water species, this would result in a 1.2-to 1.7-fold increase in carbonate production by fish in surface waters, with 0.6 °C to 2 °C of warming, respectively. Deeper waters are expected to warm more slowly, so only ~1.1-1.2-fold increases in carbonate production would be expected for fish experiencing ~0.3-0.6 °C warming at ~1,000 m depth by the end of the 21st century (IPCC Panel, 2014). This difference in the rate of warming depending on depth may be particularly relevant to mesopelagic fish, which have been implicated in driving an upward alkalinity pump (Roberts *et al.*, 2017). This involves precipitation of carbonates at depth and excretion near the surface, facilitated by the large, vertical daily migrations of mesopelagic species (Roberts

et al., 2017). As precipitation in mesopelagic fish occurs at a depth predicted to undergo smaller increases in temperature than the shallows where carbonates dissolve, ocean warming may reduce the amount of alkalinity brought to the surface by mesopelagic fish. This depends on whether larger increases in temperature at the surface reduce dissolution of carbonates enough to offset increases in carbonate production associated with smaller increases in temperature at depth.

Overall, it appears that temperature influences carbonate excretion rate in lumpfish at cold temperatures in a similar manner to other fish at higher temperatures. However, the effect of temperature on carbonate production in lumpfish seems to be stronger in the upper half of their temperature range. We believe this is because of a temperature-associated change in behaviour, which is characterised by less time spent utilising energy-saving attachment to surfaces at higher temperatures. The Q_{10} measured between 2 °C and 7°C of 3.50 may be more broadly applicable to cold water species and therefore more useful to include in future modelling attempts, although there is likely to be significant inter-species variation. Research on further species is necessary to understand whether a Q_{10} of 3.50 is truly representative of carbonate production in cold water species, and whether the heightened sensitivity to temperatures above 7 ° is due to the aforementioned behavioural irregularities.

Chapter 3: The effects of feeds enhanced with calcium salts on physiology and CaCO$_3$ production in freshwater rainbow trout

Statement

The work in this chapter was conducted in collaboration with Harriet Goodrich, a QUEX-funded PhD student at the University of Exeter, supervised by Rod Wilson. Feed design was by Harriet Goodrich and Rod Wilson, with titration of feeds to ascertain buffering capacity conducted by Harriet Goodrich. As well as to investigate the handling of dietary calcium by freshwater fish, blood chemistry analysis at 24 hours post feed was conducted to add depth to the measurements already made by Harriet Goodrich of fish that were starved and at 48 hours post-feed, which are not presented here. All other work presented here, including physical production of the feeds used in this experiment, and all data, are my own.

Introduction

In marine teleost fish, the primary function of intestinal carbonate precipitation is thought to be osmoregulation (Wilson *et al.*, 2009; Grosell, 2019), but it has also been suggested that CaCO$_3$ precipitation acts to limit excess Ca^{2+} absorption. If excreted renally, Ca^{2+} absorption may overwhelm the kidney and lead to renal stone formation (nephrocalcinosis) due to the low urine flow rates of marine fish (Wilson and Grosell, 2003). In addition to high concentrations of Ca^{2+} imbibed in seawater, predatory fish tend to consume prey whole, which can lead to a significant increase in calcium ingestion in the case of prey with bony calcium phosphate (Ca$_3$(PO$_4$)$_2$) skeletons or CaCO$_3$ shells.

The stomachs of teleost fish are acidified by the secretion of hydrochloric acid (HCl) into the stomach by oxyntopeptic cells, reducing stomach pH to ~2-4, the optimal pH for digestive enzymes (Johnston *et al.*, 2007). This secretion of H$^+$ into the stomach must be accompanied by an equimolar secretion of HCO$_3^-$ into the blood to maintain intracellular acid-base balance of oxyntopeptic cells. The release of HCO$_3^-$ into the blood causes the 'post-prandial alkaline tide', elevating blood pH (Bucking and Wood, 2008; Cooper and Wilson, 2008). Blood alkalinisation impairs enzymatic activity, gas, and ion transport (Evans, Piermarini and Choe, 2005), so the excess HCO$_3^-$ must be excreted to restore blood pH. The site for excretion of HCO$_3^-$ generated by gastric H$^+$ secretion appears to

depend on meal size, as consumption of a ration equal to 5 % of body mass was recorded to increase net base excretion at the gills (Bucking and Wood, 2008), while consumption of a 1 % ration was not (Cooper and Wilson, 2008). The intestine has been proposed as a likely candidate for non-branchial HCO_3^- excretion (Cooper and Wilson, 2008), as the intestine is already known to secrete HCO_3^- to facilitate osmoregulation in marine fish.

Dietary $Ca_3(PO_4)_2$ and $CaCO_3$ have high buffering capacity, meaning that they resist changes in pH. This means that in order to reach the optimal pH for digestion, a greater volume of acid must be secreted into the stomach, mirrored by an equimolar increase in HCO_3^- secretion into the blood. This results in the energetic cost of digesting the meal increasing, as digestion of $CaCO_3$ and $Ca_3(PO_4)_2$ does not provide the animal with any energy, while the processes of acid secretion and HCO_3^- excretion consume energy (Goodrich *et al.*, in review). The energetic cost of digesting and assimilating a meal is represented by its specific dynamic action (SDA), and usually accounts for 10-20% of the energy content of the meal (Andrade *et al.*, 2005; Fu, Xie and Cao, 2005; Secor, 2009). Larger SDA values are indicative of greater costs of digestion and less absorbed energy available for growth (Jobling, 1994). The inclusion of calcium salts with high buffering capacity into the diets of fish is likely to affect the duration and/or magnitude of the SDA independently from the nutritional value of the meal. This has implications for predatory fish in the wild which eat prey whole such as other fish that have a calcium phosphate endoskeleton or invertebrates that have a calcium carbonate exoskeleton or shell. It is also relevant to fish in aquaculture, where addition of bonemeal to feeds as a source of phosphate may result in an increase in energetic cost of digestion, leaving less energy available for growth. If shelled invertebrates make up part of the diet of fish in aquaculture, it is likely that at least some $CaCO_3$ shell remains, even if attempts are made to remove these.

In addition to their buffering capacity, the dissolution of $Ca_3(PO_4)_2$ and $CaCO_3$ in the stomach provides the fish with an abundance of Ca^{2+}. In the acidic conditions of the stomach, HCO_3^- and/or CO_3^{2-} released by the dissolution of $CaCO_3$ are converted to CO_2 which enters the blood due to the gas permeability of epithelia. Ingestion of $CaCO_3$ therefore provides the intestine with an abundance of Ca^{2+}. Calcium phosphate also dissolves in the stomach, providing an abundance of

Ca^{2+}, but also its counter ion (PO_4^{3-}), in contrast to $CaCO_3$. Fish require a certain amount of Ca^{2+} to live, but consumption of whole prey likely provides more Ca^{2+} than fish require, and the excess must therefore be excreted. As feeding events cause a high concentration of calcium to enter the digestive tract at a single timepoint, it seems likely that unchecked Ca^{2+} absorption may lead to the kidney being overwhelmed when fish are in seawater, due to low urine flow rates (Wilson and Grosell, 2003). Hypoosmotic water is readily available to fish inhabiting freshwater, but consumption of freshwater to aid in renal calcium excretion would likely lead to losses of other ions, and nephrocalcinosis is also observed in freshwater teleosts (Kent *et al.*, 2020), highlighting the dangers of renal Ca^{2+} excretion even in a hypoosmotic environment.

The Ca^{2+} concentration of seawater (~10 mM) represents part of the osmotic challenge faced by marine fish (Hwang and Lin, 2013). Due to living in hyperosmotic seawater, marine must fish drink seawater to offset osmotic losses and must manipulate its ion concentrations within the gut to absorb water. In marine fish the gills are the main site of Na^+ and Cl^- excretion (Hwang and Lin, 2013), while the kidneys handle the majority of Mg^{2+} and SO_4^{2-} excretion (McDonald and Grosell, 2006; Kato *et al.*, 2009; Islam *et al.*, 2013). Water absorption in the intestine is facilitated by three main processes: solute-coupled water absorption (facilitated by NaCl cotransport from the intestine), Cl^-/HCO_3^- exchange and alkaline precipitation of $CaCO_3$ and $MgCO_3$ (see chapter 1 for more details). The intestine is generally impermeable to divalent ions, so in the absence of precipitation, these would accumulate in intestinal fluid and impair water absorption as other ions and water are absorbed. Precipitation of intestinal carbonates results in a reduction in luminal osmolality of 70-100 mOsm/l (Wilson, Wilson and Grosell, 2002; Grosell *et al.*, 2009), facilitating water absorption, which can only occur against an osmotic gradient of 35 mOsm/l (Genz, Taylor and Grosell, 2008).

The calcium-sensing receptor (CaSR) is thought to have evolved in the marine environment to facilitate osmoregulation (Loretz, 2008), and is expressed in endocrine tissues, as well as ion-transporting tissues such as the intestine (Gregório and Fuentes, 2018). Interestingly, CaSR expression in the anterior intestine of sea bream is significantly higher in fed compared to unfed fish (Gregório and Fuentes, 2018). When measured *ex vivo* in Ussing chambers,

altering apical saline Ca^{2+} concentration led to proportional increases in HCO_3^- secretion, i.e, a ~2-fold increase in Ca^{2+} concentration led to a ~2-fold increase in HCO_3^- secretion (Gregório and Fuentes, 2018). European flounder intestines perfused *in vivo* with 4-fold higher Ca^{2+} concentration apical saline also show strong evidence for increased HCO_3^- secretion in response to high Ca^{2+} in the gut lumen, exhibiting a 1.57-fold increase in total HCO_3^- equivalents, and a 7-fold increase in solid carbonate excretion (Wilson, Wilson and Grosell, 2002). This strongly suggests that fish are able to mediate HCO_3^- secretion in order to precipitate ingested Ca^{2+}. As Ca^{2+} ingested as part of a calcium salt enters the intestine in solution as Ca^{2+}, it would appear that this could stimulate CaSR in the same way as elevating Ca^{2+} concentration of apical saline in Ussing chambers.

In a previous study, the authors anecdotally observed intestinal $CaCO_3$ precipitates excreted in the faeces of freshwater rainbow trout that resembled those excreted by marine fish. We therefore aimed to explore this further using diets supplemented with calcium salts to investigate the effects of dietary calcium on intestinal $CaCO_3$ formation, but in the absence of the source of Ca^{2+} within the ingested hyperosmotic seawater previously studied in marine fish. We proposed that all calcium salts ingested by the fish would be dissolved in the acidic stomach, releasing free Ca^{2+} ions, and that this would be followed by precipitation of these Ca^{2+} ions in the intestine as $CaCO_3$. We predict that the cellular processes associated with HCO_3^- secretion in seawater fish will be 'switched on' in order to precipitate excess dietary calcium, preventing excessive absorption into the blood. The calcium salts chosen to supplement the diets of freshwater trout included calcium phosphate (found in the bone of fish prey) and calcium carbonate (found in the exoskeleton/shell of calcified invertebrates). These minerals have high acid-buffering capacity, so we also hypothesised that diets supplemented with these salts would require greater rates of gastric HCl secretion for digestion, and therefore a more pronounced alkaline tide (greater blood HCO_3^- and pH) at 24 hours post-feed.

Materials & methods

Fish husbandry pre-experiment

Juvenile rainbow trout (*Oncorhynchus mykiss*) (n= 30; body mass: 127.2 ± 3.3 g) were obtained from Houghton Springs Fish Farm, Dorset, UK. Upon arrival (Aquatic Resources Centre, University of Exeter), fish were stored in a ~500 L stock tank provided with fully aerated freshwater in a miniature recirculating system, at 15 °C. Trout feed (Acclimer), equal to 1% of the total mass of the fish in the tank, was provided three times per week. Fish were starved for seven days prior to 30 individuals being moved to individual chambers for experimentation. Fish were then offered a 2.5% body mass ration for 4 weeks, at the end of which all fish consumed >95% of the offered ration.

Calcium salt diets

Fish were fed Skretting 4.5 mm Horizon trout pellets (Skretting, UK) supplemented with a calcium salt to represent the calcium content of fish bone ($Ca_3(PO_4)_2$), invertebrate shell ($CaCO_3$) or a non-buffering control ($CaCl_2$). Pure $Ca_3(PO_4)_2$ requires 10.25 times as much acid per mg to reach the approximate pH (3.5) of rainbow trout stomach chyme during digestion. The wet mass of bone constitutes ~16.3% of the total wet mass of a fish (Cameron, 1985). For each 100 mg of pellet feed, 19.5 mg of bone would be required to represent the normal amount in a whole fish. Therefore 1.9 mg of pure $Ca_3(PO_4)_2$ was added per 100 mg of pellet feed to represent the buffering capacity of the bone content of a fish. This is likely to be similar to the $Ca_3(PO_4)_2$ content of a fish prey, as bone is not pure $Ca_3(PO_4)_2$. The diet containing additional $Ca_3(PO_4)_2$ is hereby referred to as the phosphate diet. In order to ensure each diet contained equimolar Ca^{2+}, 1.84 mg of $CaCO_3$ or 2.7 mg of $CaCl_2$ (dihydrate) were added per 100 mg of pellet feed to make up the $CaCO_3$ or $CaCl_2$ diets (hereby the carbonate or chloride diets), respectively. To make up pellets of each diet, 100 grams of Skretting 4.5 mm Horizon trout pellets were ground to a fine powder using a blender. To the ground pellet, either 1.9 g of $Ca_3(PO_4)_2$, 1.84 g of $CaCO_3$, or 2.7 g of $CaCl_2$ (dihydrate) were added and mixed. After addition of 70 ml ultrapure water to form a paste, the mixture was added to a syringe. The mixture was then extruded from the syringe, and the resulting cylinders of feed paste were cut with a scalpel to form pellets of a similar size to the original pellets. These were then dried for 24 hours at 70 °C to form the final dry pellet. The buffering capacity of each feed was

measured by titration to pH 3.5 with 228.3, 320.0 and 550.0 µmoles of HCl required to titrate 1 g of the chloride, phosphate and carbonate feeds respectively.

Ion fluxes to external water and faeces collection

Thirty fish were isolated in ~20 L tanks. These were provided with fully aerated freshwater from the same miniature recirculating system as the stock tank, with additional aeration in each tank. Prior to feeding, flow to the tank was removed to ensure that no pellets were accidentally flushed from the tank. Fish were fed a 2.5% body mass ration, once per week, of one of the three treatment diets, or the unmodified pellet diet, and any pellets not eaten after 15 minutes were counted and removed. Water flow was then restored and any pellet detritus from eating was siphoned out.

After 4 weeks of acclimation to experimental conditions, we began experimentation in order to understand post-prandial ion fluxes related to the presence of calcium salts in the diet. Fish were fed as outlined above, and after water flow had been restored for 15 minutes (during which time food detritus was removed), water flow was again stopped. Following this, 55 ml water samples were removed from the tanks to allow for analysis of HCO_3^- and Ca^{2+} content. After 12 hours a further 55 ml water sample was removed, and any remaining food detritus removed (no faeces were excreted within 12 hours post-feed). Water flow was then restored for 15 minutes to allow for exchange of the water in the tank. After 24 hours faeces were collected from the tank into a sample tube at the same time as 55 ml water samples were taken. This process was repeated every 12 hours until 96 hours post-feed. As additional faeces were produced by some fish after 96 hours, faeces collection continued until 132 hours post feed, but additional water samples were not taken.

Unfortunately, due to technical issues and COVID-restrictions during lockdowns, we were so far unable to analyse water HCO_3^- or Ca^{2+} concentrations in order to calculate fluxes to or from the external water. We hope to be able to measure water Ca^{2+} concentration for future publication.

Faecal precipitate analysis:

Precipitate preparation and analysis was conducted as outlined in chapter 2.

Following the double-endpoint titration to determine precipitate CO_3^{2-} content, samples were re-acidified using an amount of HCl equal to the amount of NaOH

that was used to return pH to the starting pH. This meant that any precipitates that formed during base titration were re-dissolved, and dissolved ion concentrations could be measured, specifically for concentrations of Ca^{2+}, Mg^{2+} and PO_4^{3-}. Unfortunately, as mentioned above for water ion concentrations, we were so far unable to measure these due to COVID-restrictions and lack of availability of the ion chromatography system. We also hope to be able to measure these for future publication.

We calculated the percentage of additional Ca^{2+} consumed as a result of calcium salt addition to the diet, that was excreted as $CaCO_3$ in the faeces (assuming 100% of titrated CO_3^{2-} was $CaCO_3 - CaCO_3\%$) as follows:

$$CaCO_3\% = \frac{E - uE}{C} \times 100$$

Where E is the total amount of $CaCO_3$ excreted by a fish (in µmoles) in the 144 hours following consumption of a feed supplemented with a calcium salt, uE is the mean total amount of $CaCO_3$ excreted by all fish (in µmoles) in the 144 hours following consumption of the unmodified diet, and C is the total amount of Ca^{2+} (in µmoles) added to the diet in the form of a calcium salt.

Blood, chyme, and intestinal fluid chemistry:

In order to investigate the effects of dietary calcium salts on digestive physiology and blood chemistry, samples of blood, stomach chyme and intestinal fluid were taken from fish 24 hours after feeding. After a 7 day fast, fish were fed a ration of 2.5% of their body mass (measured when they were moved into individual tanks at the start of the experiment). As the fish had grown since then, the average ration was 2.19% (n=24 (6 per diet), se=0.013).

At 24 hours post-feed, fish were anaesthetised in their tanks using benzocaine (75 mg/L). Fish were observed until they lost equilibrium and did not respond to a light tail pinch. Fish were then transferred to a 'gill irrigation' system dosed with benzocaine (30 mg/L) in order to provide a constant flow of aerated water over the gills, aimed at approximately matching normal *in vivo* ventilatory flow, while maintaining anaesthesia. Without irrigation of the gills, blood O_2 can be depleted, while CO_2 and lactic acid can accumulate, affecting blood gases and acid-base chemistry. When a stable gill ventilation was achieved, blood was then attained via caudal vessel puncture using a heparinised 1 ml syringe (using 5,000 U/mL

sodium heparin: Monoparin). This was immediately followed by euthanasia of the fish by pithing to destroy the brain. Fish were then weighed and dissected to collect stomach and intestine contents, which were centrifuged to separate fluids and solids for analysis.

Blood, stomach, and intestinal fluid pH were measured using an Accumet CP-620-96 MicroProbe connected to a Hanna HI 8424 meter. Blood PO_2 was measured using a Strathkelvin 1302 electrode and 781 meter. A haematocrit centrifuge (Hawksley) was used to isolate plasma, and a haematocrit reader was used to measure haematocrit. Plasma and intestinal fluid TCO_2 was measured using a Mettler Toledo 965 carbon dioxide analyser, which were used to calculate plasma and intestinal PCO_2 and HCO_3^- in combination with pH using a rearranged Henderson-Hasselbach equation and values for pK_1 and solubility in the case of blood (Boutilier, Heming and Iwama, 1984), or the apparent pK_2 of the HCO_3^-/CO_3^{2-} equilibrium (Wilson, Wilson and Grosell, 2002). Any leftover plasma was snap frozen and stored at -80 °C before analysis of total lactate and glucose concentration (YSI 2900D Biochemistry Analyzer). We also hope to analyse plasma and intestinal fluid ion concentrations in the future by ion chromatography (Dionex ICS 1000 and 1100).

Statistics:

All statistical analyses were performed in Graphpad Prism 9. Shapiro-Wilk tests were used to establish whether data were normally distributed. Following this, non-parametric Kruskal-Wallis tests (when data were not normal) and parametric one-way ANOVAs (when data were normal) were conducted to detect statistically significant differences between groups.

Results:

Ration (as % body mass) fed to fish

The mean ration actually consumed ranged from 2.16 to 2.25 % of body mass, but there were no statistically significant differences between dietary treatments (Figure 7).
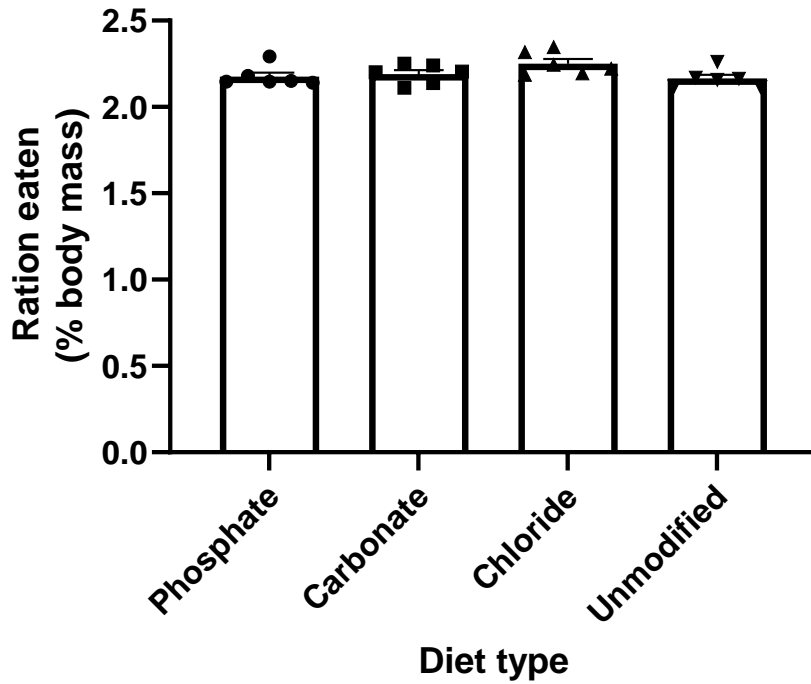
Figure 7: The ration eaten for of each diet fed to fish in order to measure blood and gastrointestinal chemistry parameters at 24 hours post-feed. Fish were offered 2.5% of their total body mass at the start of the experimental period. Data shown here are calculated from the total mass of the feed that they consumed 24 hours prior to euthanasia, and their final body mass following this. Data are mean ± standard error. Symbols represent the ration fed to individual fish. There were no statistically significant differences between groups.

Blood chemistry parameters 24 hours post-feed

The two individuals in which plasma lactate was measured at >1.5 mmol/L were excluded from analysis of other variables, as elevated lactate in the blood indicates anaerobic production of metabolic acid that affects blood gases and acid-base chemistry. There were no statistically significant differences between groups. Blood pH appeared reduced by ~0.1 in fish fed the chloride diet, compared to all other diets, and starved fish. Blood PCO_2 appeared to be slightly reduced in fish that were starved, compared to fed fish. There were no differences of note for any of the other measured or calculated parameters.
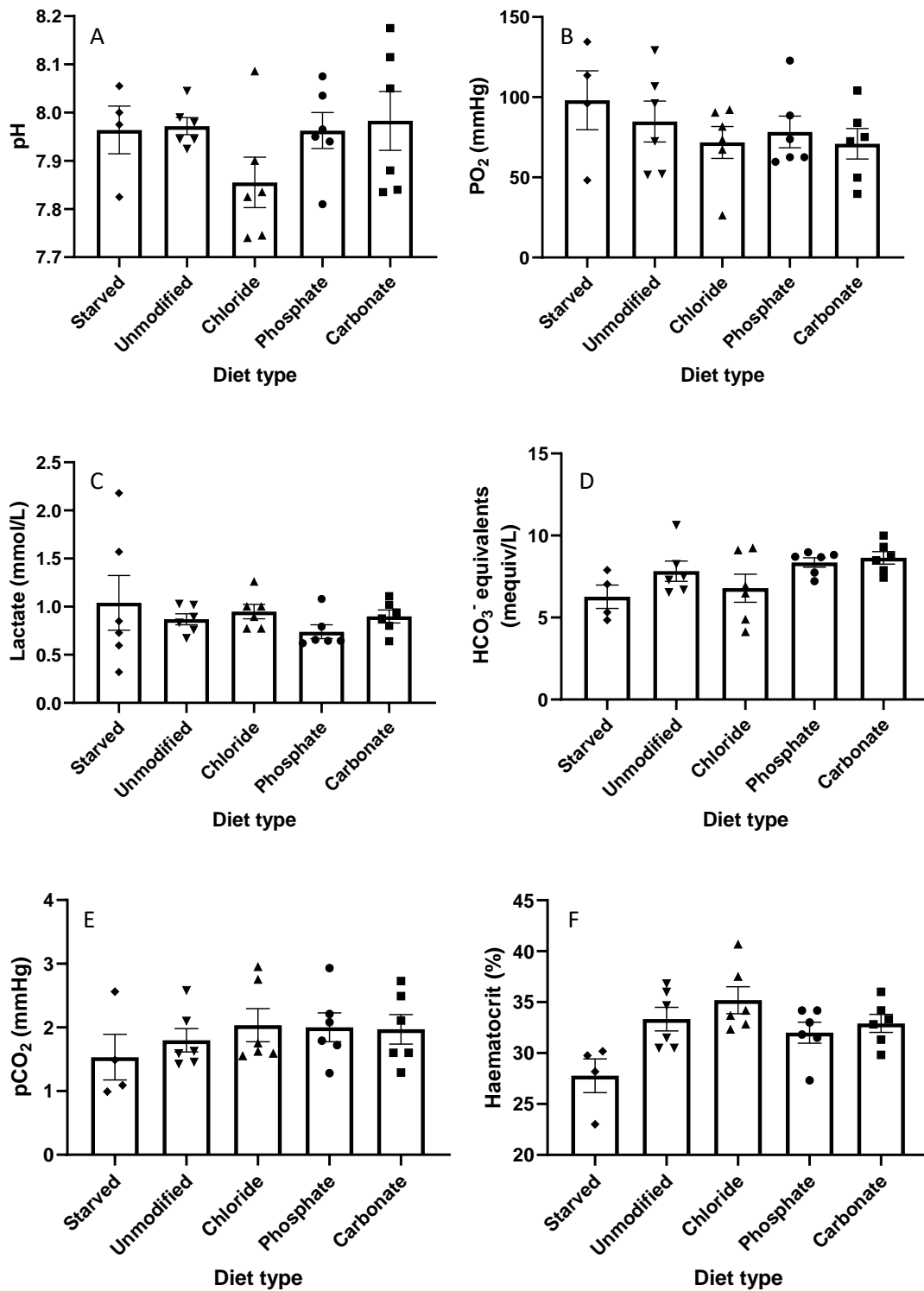
Figure 8: Blood chemistry parameters of rainbow trout 24 hours after being fed 2.5% body mass of a feed supplemented with $Ca_3(PO_4)_2$ (phosphate), $CaCO_3$ (carbonate), $CaCl_2$ (chloride) or a feed not supplemented with any calcium salts (unmodified). Blood pH (A), PO_2 (B), lactate (C) and Haematocrit (F) were directly measured, whilst HCO_3^- equivalents (D) and PCO_2 (E) were calculated (see methods for details). Statistically significant differences, if any, are represented by different letters. The bars represent mean ± standard error, with symbols representing individual measurements. The two fish with highest lactate (indicating interference from some anaerobic generation of metabolic acid during anaesthesia) in the starved group were removed from analysis of other blood chemistry parameters. There were no statistically significant differences between groups.

Gastrointestinal chemistry parameters 24 hours post-feed

There were no significant differences in gastrointestinal chemistry between groups. Diets modified with calcium salts with buffering capacity did appear to affect stomach pH, with fish fed the carbonate and phosphate diets having ~0.4 and ~0.2 higher stomach pH, respectively, than fish fed the chloride and unmodified diets. The feed with highest buffering capacity (carbonate) included the three highest stomach pH measurements, and four of the highest six pH measurements. Intestinal pH appeared to be slightly lower in fish fed the phosphate diet (0.1 – 0.2), compared fish fed the carbonate, chloride, and unmodified feeds, respectively. Fish fed the phosphate and unmodified diets exhibited lower intestinal HCO_3^- equivalent concentration (~5 – 10 mequiv/L), than fish fed the carbonate and chloride diets.
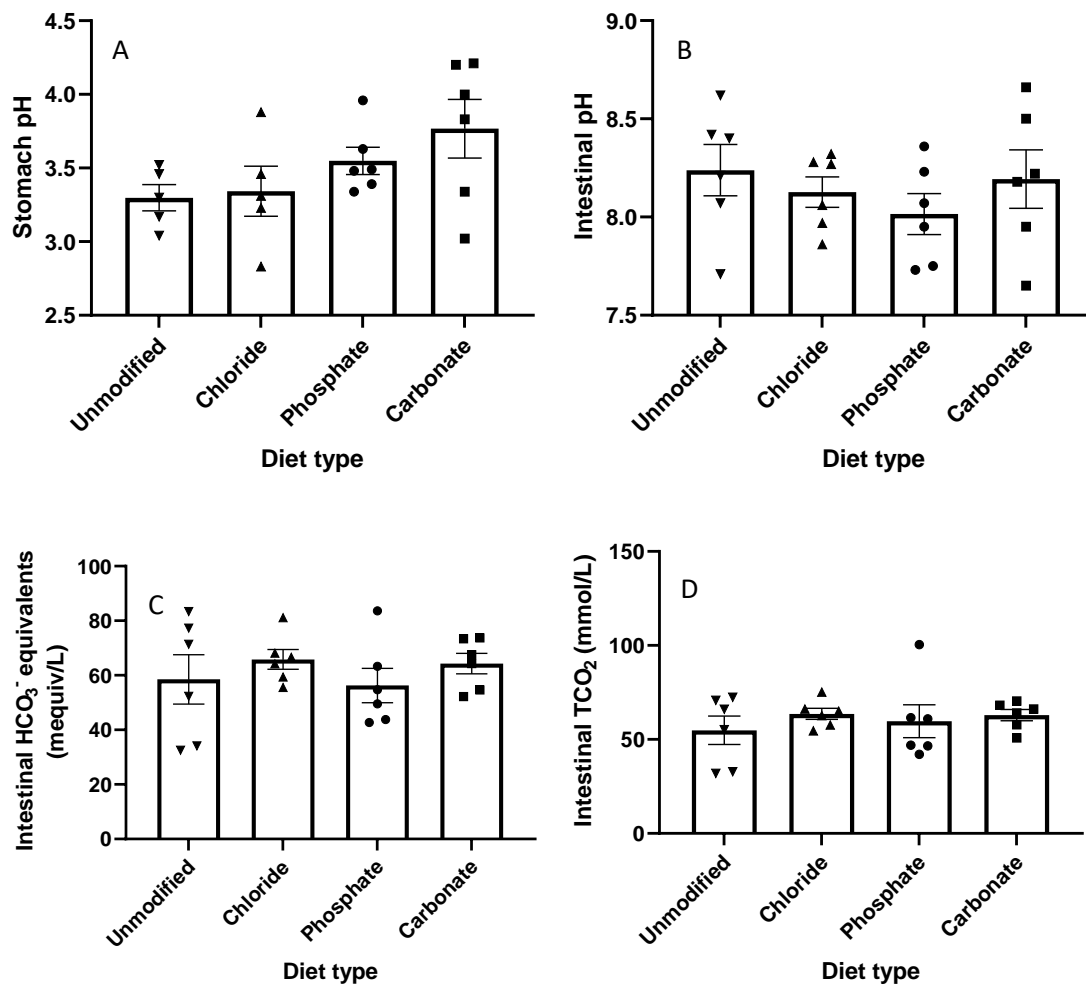
Figure 9: Gastrointestinal chemistry parameters of rainbow trout 24 hours after being fed 2.5% body mass of a feed supplemented with $Ca_3(PO_4)_2$ (phosphate), $CaCO_3$ (carbonate), $CaCl_2$ (chloride) or a feed not supplemented with any calcium salts (unmodified). Stomach pH (A), intestinal pH (B) and intestinal TCO2 (D) were directly measured, whilst intestinal HCO3- equivalents (C) was calculated (see methods for details). The bars represent mean ± standard error, with symbols representing individual measurements. There were no statistically significant differences between groups.

Excretion of Ca^{2+} as intestinal precipitated $CaCO_3$

Compared to the unmodified diet, rainbow trout fed the carbonate diet (P=0.0035) and chloride diet (P=0.0098) produced significantly more carbonate over the collection period. Fish fed the phosphate, carbonate and chloride diets excreted ~1.7-, ~4.3- and ~3.3-fold more carbonate, respectively, than those fed the unmodified diet. In all diets >80% of carbonates were excreted between 12- and 84-hours post-feed, with most of the difference between treatments occurring between 12- and 48-hours post-feed. Diets are shown in order of pH buffering capacity, with the unmodified and chloride diets having equal pH buffering

capacity. The relationship between dietary buffering capacity and carbonate excretion rate is clearly not linear.

Figure 10: Cumulative CO_3^{2-} excreted as precipitate over time by rainbow trout fed 2.5% body mass of a feed supplemented with $Ca_3(PO_4)_2$ (phosphate), $CaCO_3$ (carbonate), $CaCl_2$ (chloride) or a feed not supplemented with any calcium salts (unmodified). Red circles, blue squares, green upward pointing triangles and back downward pointing triangles represent the mean (± standard error) amount of carbonate excreted in faeces up to the time point indicated on the x axis by fish fed the phosphate, carbonate, chloride, and unmodified feeds, respectively. Some error bars are not visible as they are small enough to be obscured by data points.

Figure 11: CO_3^{2-} excreted as precipitate by fish fed 2.5% bodyweight of a feed supplemented with $Ca_3(PO_4)_2$ (phosphate), $CaCO_3$ (carbonate), $CaCl_2$ (chloride) or a feed not supplemented with any calcium salts (unmodified). Red circles, blue squares, green upward pointing triangles and black downward pointing triangles represent the mean (± standard error) amount of carbonate excreted in faeces in the 12-hour period preceding the time post-feed indicated on the x axis by fish fed the phosphate, carbonate, chloride, and unmodified feeds, respectively.

Figure 12: Total carbonate excreted by rainbow trout fed 2.5% body mass of a feed supplemented with $Ca_3(PO_4)_2$ (phosphate), $CaCO_3$ (carbonate), $CaCl_2$ (chloride) or a feed not supplemented with any calcium salts (unmodified) after 144 hours. Data are mean ± standard error, with individual values shown by symbols. Statistically significant differences are indicated by different letters.

Figure 13: Carbonate excreted by rainbow trout fed 2.5% bodyweight of a feed supplemented with $Ca_3(PO_4)_2$ (phosphate), $CaCO_3$ (carbonate), $CaCl_2$ (chloride) after 144 hours, as a proportion of the total additional calcium consumed as a result of diet supplementation. Data are mean ± standard error, with individual values shown by symbols. There were no statistically significant differences between diets.

Discussion:

These data suggest that addition of calcium salts to the diet of freshwater rainbow trout, or indeed feeding in general, did not significantly affect blood chemistry at 24 hours post-feed, compared to starved fish. This is in agreement with similar experiments on rainbow trout voluntarily fed 5% or 1% body mass rations of diets without calcium salt supplementation, which have reported no significant differences in blood pH or HCO_3^- concentration at 24 hours post-feed (Bucking and Wood, 2008; Cooper and Wilson, 2008). These previous studies have suggested that the peak of the alkaline tide is at around 6 hours post-feed, and that by 24 hours post-feed blood pH and HCO_3^- have returned to pre-feeding levels, so it is possible that we sampled fish too late to be able to detect the alkaline tide. The addition of buffering calcium salts may still have affected the length and/or duration of the alkaline tide, as 18 hours after feeding on a 5% bodyweight ration, blood pH had already returned to pre-feeding levels (Bucking and Wood, 2008). As our fish were fed on less than half the ration size of those in the aforementioned study, it is not surprising that we did not detect an alkaline tide, at least in fish fed the unmodified diet. Haematocrit did appear to be elevated in fed fish, as would be expected due to the metabolic demand of digestion, as additional red blood cells aid in oxygen delivery.

It is possible that over-ventilation of some fish during blood sampling could have contributed to the lack of differences in blood pH observed between groups. If the gills were ventilated too strongly, this could have resulted in the simultaneous removal of more CO_2 and addition of oxygen to the blood. This removal of CO_2 from the blood would artificially elevate pH. This is likely to have a more profound effect on the blood pH of individuals with lower concentrations of blood HCO_3^- as the associated reduced buffering capacity means that pH will respond more strongly to removal of CO_2. As PCO_2 and HCO_3^- were slightly lower in starved fish (~0.3-0.5 mmHg and ~0.5-2.4 mequiv/L, respectively – see figure 7D & E), I propose that overventilation of these fish may have resulted in elevation of blood pH and masking of alkaline tide-related elevations in blood pH, if there were any. This is the opposite of what is observed when fish blood is sampled without gill ventilation and CO_2 accumulation in the blood occurs. In the case of high CO_2, fish accumulate HCO_3^- in the blood, which can buffer CO_2 that accumulates in the blood after termination. When blood pH is measured after termination without

ventilation, this gives results that suggest hypercapnia exposure elevates blood pH relative to control fish acclimated to normal CO_2 levels (Fivelstad *et al.*, 1998; Brauner *et al.*, 2019). However, this is more likely caused by the CO_2 accumulated in the blood of hypercapnic fish being buffered by HCO_3^- to a greater degree than it is in normocapnic fish, resulting in higher pH of hypercapnic fish.

Additionally, because of the removal of blood data from two of the starved fish due to high levels of lactate (>1.5 mmol/L – see figure 7C), the low n (4) for this treatment (following exclusion of these 2 individuals) may have made our data for baseline before feeding less reliable than the measurements of fish fed the experimental and unmodified diets (where n=6). For the reasons stated here and in the previous paragraph, from here we will mostly refer to differences in blood chemistry between diet types, rather than comparison with starved fish.

The high variability we observed in most measured parameters could be explained by insufficient mixing of added calcium salts into the diets, resulting in higher concentrations of salts in some pellets than others. This would result in some fish fed the phosphate and carbonate feeds consuming a diet with higher or lower buffering capacity diet (and calcium content), than intended, with consequences for gastrointestinal and blood chemistry. However, this seems unlikely as results were not noticeably more variable in blood chemistry between buffering feeds (carbonate and phosphate), compared to non-buffering feeds (chloride and unmodified) or starved fish (see figure 7). Carbonate excretion values were also very variable, with the greatest proportional difference in total carbonate excreted, per kg of fish, in the unmodified feed (~4.6-fold between highest and lowest amount excreted).

Interestingly, the fish fed the $CaCl_2$ diet experienced a slightly lower blood pH and plasma HCO_3^- (~0.1 pH units and ~1-2 mequiv/l, respectively – see figure 7A and D) at 24 hours post-feed (although this was not statistically significant – P=0.4125) compared to the other diets, despite consuming a slightly greater ration than those fed the other diets. A possible explanation is that the additional Cl^- provided to the intestine by the $CaCl_2$ diet could create a more favourable electrochemical gradient to fuel HCO_3^- secretion from the blood to the intestine, as this is via Cl^-/HCO_3^- exchange (Kurita *et al.*, 2008; Taylor, Mager and Grosell, 2010). This could have resulted in greater rates of secretion of HCO_3^- from the blood to the intestine, which would explain the reduced blood HCO_3^-

concentration and pH compared to the phosphate, carbonate, and unmodified diets. Indeed, the plasma HCO_3^- in fish fed the chloride diet was very similar to the starved fish, suggesting that bicarbonate secretion promoted by extra dietary chloride during the intestinal phase of digestion (~24 h) helped the fish recover from the alkaline tide induced by the gastric phase of digestion (<24 h).

Although the differences that we measured in stomach pH were not statistically significant, fish fed the buffering feeds did generally appear to have slightly less acidic stomachs than those fed non-buffering feeds (see figure 8A). There are two main ways in which this could affect the results of other parameters measured in this study. It is possible that the fish secreted a similar amount of acid into the stomach regardless of diet consumed, and that this resulted in the stomach pH that we measured. In this case, there would be little difference in alkaline tide between diets, as similar amounts of HCl secretion between diets would result in equimolar HCO_3^- secretion into the blood. However, a higher gastric pH (~0.2-0.4 pH units in buffered feed) could result in less efficient digestion due to sub-optimal pH for digestive enzymes, and therefore result in less energy and nutrients being absorbed. Alternatively, more HCl may have been required to reduce stomach pH to the levels measured. This would have increased the alkaline tide (perhaps before the 24 hours post-feed sampling point), with both the additional gastric HCl secretion and subsequent HCO_3^- excretion from the blood entailing higher energetic cost. Either way, this could result in less energy being available for growth, leading to a reduced calcium requirement for growth. If less calcium is required for growth, more must be excreted and therefore more Ca^{2+} may be available in the intestine for precipitation, potentially resulting in greater carbonate excretion from fish consuming buffered feeds.

It is important to note that the amount of gut carbonate excreted by fish within each collection period was extremely variable, which explains our high error values and lack of statistically significant differences between some diets, despite high absolute differences between mean values at particular time points. As already mentioned, in fish fed the unmodified feed, we observed a ~4.6-fold difference in total carbonate excretion (117 vs. 544 µmol/kg) between individual fish that excreted the most and least carbonate, which was a greater proportional difference than was observed in any of the modified feeds. The amount of carbonate measured in faeces accounted for 27.6%, 19.5% and 9.6% of the extra

Ca^{2+} consumed in the carbonate, phosphate, and chloride diets, respectively (see figure 12). The remaining Ca^{2+} could have been absorbed, excreted as precipitate and subsequently dissolved, or excreted via a number of pathways. There did not appear to be linear effect of dietary buffering capacity on carbonate excretion, as the phosphate diet (with intermediate buffering capacity compared to the chloride and carbonate diets) induced fish to produce conspicuously less carbonate than those fed either the chloride or carbonate diets. This suggests that differences in carbonate production may be related to how the anions provided by calcium salts are processed by the gut.

Some of the absorbed Ca^{2+} would have been utilised by the fish for growth, especially in processes such as bone formation. Intestinal fluid would also have contained Ca^{2+}, and we hope to measure its concentration at a later date. There is also likely to have been an increase in urinary Ca^{2+} excretion via the kidney, as urinary Ca^{2+} excretion has been observed to increase ~1.4-fold after feeding (Bucking, Landman and Wood, 2010). It is also possible that branchial excretion of Ca^{2+} occurred after feeding, although the gills are usually involved in Ca^{2+} uptake in freshwater (Kurita *et al.*, 2008; Hwang and Lin, 2013). Some $CaCO_3$ in the faeces of fish is likely to have dissolved between excretion and collection, resulting in increases in Ca^{2+} concentration in the water in tanks. We hope to measure Ca^{2+} concentrations at the beginning and end of each flux period in future, which will help to quantify the amount of Ca^{2+} excreted, but not contained in collected carbonates. This will in turn allow us to quantify the amount of Ca^{2+} absorbed from the meal by each fish, but not the pathway by which the Ca^{2+} reached the external water, as Ca^{2+} excreted via the gills, kidney, intestinal fluid, or carbonates dissolved post-excretion would not be differentiable via our methods.

Rectal catheterisation would provide a more comprehensive picture of excretion of Ca^{2+} via the intestine. This would allow measurement of intestinal fluid excretion rate, Ca^{2+} excretion as precipitate (without the possibility of dissolution between excretion and collection) and Ca^{2+} in the intestinal fluid. However, keeping carbonates (within faeces) in alkaline gut fluid after excretion may result in additional precipitation, and therefore may overestimate the contribution of precipitate to Ca^{2+} excretion, while underestimating the contribution of intestinal fluid. Despite this, rectal catheterisation would be useful for quantifying the overall

contribution of the intestine to Ca^{2+} homeostasis and help to identify how much Ca^{2+} was absorbed into the body and subsequently used or excreted via the kidney or gills. Unfortunately, it is unlikely that rectal-catheterised rainbow trout would feed voluntarily, and these types of catheters would probably lose their patency (typically within 3-5 days) before the fish had sufficiently recovered their appetite following anaesthesia and surgery. So, whilst a potential solution in theory, this approach may not provide the answers desired.

The amount of carbonate that may have dissolved between excretion and collection of faeces is difficult to predict and is complicated by a number of factors. Firstly, the solubility of carbonates produced by fed fish has not been measured. Freshwater is likely to be more corrosive to carbonates than seawater, depending on alkalinity and calcium hardness. We did not measure water chemistry in this study, but the saturation state of carbonates in freshwater is likely to be <0.01 (calculated in CO2Sys, assuming salinity = 0.5 ppt, total alkalinity = 200 µmol/kg, temperature = 15 °C, PCO2 = 440 µatm). Freshwater chemistry varies depending on a multitude of factors, and in some cases Ca^{2+} may be higher than in seawater, so it is not possible to provide a representative value for freshwater (Pinheiro *et al.*, 2021). In fed fish, precipitates tend to be integrated within faeces, rather than excreted directly into the external water within a rapidly degraded mucus coating, as are carbonates produced as a result of osmoregulation in the absence of food intake in seawater fish. Therefore, the conditions within the faeces themselves may be more relevant to the dissolution of faecal carbonates than the chemistry of the water surrounding them. This is an intriguing avenue for future research, as the majority of precipitates produced within the marine environment are likely to be incorporated with faeces, and aspects such as their sinking and dissolution rates are key to understanding their contribution to ocean chemistry (Roberts *et al.*, 2017).

Fish fed the $Ca_3(PO_4)_2$ diet excreted less carbonates in their faeces than those fed either the $CaCO_3$ or $CaCl_2$ diet. There are multiple possible reasons for this, mostly explained by PO_4^{3-} entering the intestine along with Ca^{2+} from $Ca_3(PO_4)_2$ dissolution in the stomach. Because of the buffering capacity of PO_4^{3-}, the intestinal fluid of fish fed the phosphate diet resist changes in pH more than those fed the other diets, as can be seen by the slightly higher gastric pH as well as slightly lower intestinal pH exhibited in these fish (see figure 8B). It is also likely

that at least some of the $Ca_3(PO_4)_2$ that was dissolved in the stomach re-precipitated in the intestine, reducing the amount of Ca^{2+} available for carbonate formation. In seawater, phosphate adsorbs strongly to calcite and aragonite surfaces and strongly inhibits carbonate precipitation (Morse, Arvidson and Lüttge, 2007). While I am not aware of any research on the effects of phosphate in high pH, high HCO_3^- environments like the fish intestine, it seems likely that phosphate still acts to inhibit carbonate formation in this environment. It is also possible that the additional dietary PO_4^{3-} provided by the phosphate diet affected bone mineralisation. More phosphorous may be required to maximise bone mineralisation than soft tissue growth (Robinson *et al.*, 1987). If the phosphorous content of the unmodified diet fell below the threshold for maximising bone mineralisation, the phosphate diet is likely to have increased bone mineralisation, which requires Ca^{2+}. Therefore, a greater amount of Ca^{2+} may have been absorbed for the purpose of mineralisation, reducing the availability of Ca^{2+} for calcification in the gut. As mentioned earlier, the buffering capacity of the phosphate diet may have resulted in less energy available for growth, so this may result in more Ca^{2+} being available for precipitation, counteracting aforementioned factors to a certain extent. Overall, I believe the re-precipitation of $Ca_3(PO_4)_2$ and the inhibition of carbonate formation by phosphate were likely to be quantitatively the most important factors in explaining the differences in carbonate excretion measured in fish fed the phosphate diet, compared to those fed the other diets.

The differences in carbonate production between fish fed the chloride and carbonate diets are interesting, as they should have provided the same amount of Ca^{2+} to the intestine, without a counterion that is likely to precipitate under alkaline conditions. The differences in blood chemistry 24 hours post-feed may help to explain why these differences may have occurred. The less pronounced blood alkalinisation observed in fish fed the chloride diet, in combination with their lower stomach pH, suggests that the cost of digestion of this diet may have been lower than the carbonate diet. The higher cost of the digestion of carbonate diet may therefore have resulted in less energy available for growth, and therefore less calcium required for growth. Additionally, elevated HCO_3^- in the blood stimulates intestinal HCO_3^- secretion (Grosell *et al.*, 2005), so this may also have occurred in fish fed the carbonate diet, resulting in an increased availability of

both HCO_3^- and Ca^{2+} in the intestine, which may explain the greater carbonate production compared to those fed the chloride diet.

Rainbow trout have previously been recorded to increase urinary Ca^{2+} excretion by ~40% following feeding, equating to an increase in urinary Ca^{2+} excretion of 3 μmol/kg/h (Bucking, Landman and Wood, 2010). Over the peak of intestinal carbonate excretion (12-48 hours post-feed), this could account for an increase in Ca^{2+} excretion of ~108 μmol/kg, which equates to ~50% of the Ca^{2+} excreted as carbonate by fish fed the unmodified diet, assuming the precipitates were 100% $CaCO_3$. Nephrocalcinosis occurs in freshwater fish (Kent *et al.*, 2020), suggesting that despite an abundance of hypoosmotic water which has the potential to facilitate renal Ca^{2+} excretion, this still poses risks to the fish. As fish fed the calcium salt-supplemented diets had excreted Ca^{2+} as carbonate to a much greater extent than the fish fed the unmodified diet, it seems unlikely that the kidney would have increased its contribution to Ca^{2+} excretion to anywhere close to the same extent as the intestine.

The current data were obtained using freshwater rainbow trout, but an intriguing prediction can be made if we assume that analogous results would occur in seawater fish fed on similar high calcium diets. Specifically, the higher volume of carbonates excreted by fish consuming the $CaCO_3$ diet than the $Ca_3(PO_4)_2$ diet suggests that fish consuming shelled invertebrates may contribute more significantly to global marine carbonate production than those consuming vertebrate prey with a bony skeleton. This is relevant to ocean carbonate chemistry modelling, as the availability of prey species seems very likely to be important for accurately estimating carbonate production by marine fish. Marine fish that consume shelled invertebrates and excrete $CaCO_3$ in their faeces are not necessarily precipitating 'new' $CaCO_3$, in the sense that they do with carbonates produced as a by-product of osmoregulation. However, the $CaCO_3$ excreted does not have the same structure as the $CaCO_3$ that is consumed. Invertebrates with large skeletons have a low surface area: volume ratio, compared to the nano- or microscale carbonates excreted by marine fish, thus intestinal excretion of predatory fish-derived carbonates should make a greater proportion of the original prey-derived carbonate (skeleton) vulnerable to dissolution in seawater. Thus, marine fish may be effectively converting invertebrate calcium carbonate skeletal material into a more soluble mineral type

(high-Mg calcite) and with a higher surface area morphology making it much more prone to dissolution. Even in calcifying microorganisms such as coccolithophores with a similarly high surface area: volume ratio, the aragonite and calcite that make up their coccoliths/ other bodily structures are much less soluble than the high-Mg calcites produced by fish (1.95 times as soluble as aragonite; Woosley, Millero and Grosell, 2012). Therefore, $CaCO_3$ that has been consumed and excreted by fish may dissolve more quickly in the surface ocean than if left in the form it was in before consumption.

Carbonates produced by marine fish are notable for their high magnesium content of up to 54 mol% Mg^{2+} (Foran, Weiner and Fine, 2013). As this experiment took place in freshwater, there was minimal Mg^{2+} available from the environment, so there was unlikely to be significant Mg^{2+} incorporation into carbonates. In seawater-acclimated fish, Mg^{2+} is readily available in seawater, so would likely be incorporated into carbonates more readily. This is important, as elevated Mg^{2+} content increases solubility of carbonates (Morse, Arvidson and Lüttge, 2007; Purgstaller *et al.*, 2019). We would therefore expect carbonates produced by marine fish consuming similar diets to those in the present study to incorporate a greater proportion of Mg^{2+}, and therefore be more soluble. However, most freshwater is much more corrosive to carbonates than seawater, which could potentially outweigh the effect of Mg^{2+} on carbonate solubility, so carbonates may dissolve more quickly when exposed to freshwater. However, despite being defined by its low salinity, freshwater has incredibly variable inorganic chemistry, with both calcium concentration and alkalinity (that together define the Ω_{CaCO3}) varying by more than 1,000-fold both regionally and globally (Pinheiro *et al.*, 2021), and can even be higher than seawater in places where the local geology is dominated by calcareous rocks. So even this presumption about high solubility of fish gut carbonates in freshwater is context specific. Furthermore, as already mentioned, excretion within faeces may protect carbonates from external conditions, so the internal chemistry of faeces may be more relevant to predicting dissolution than water chemistry.

A fascinating outcome of this study is that intestinal HCO_3^- concentration of fed freshwater-acclimated rainbow trout was strikingly similar to that measured in fed seawater-acclimated rainbow trout (~61 mequiv/L across all diets in this study vs. ~68 mequiv/L in fed seawater-acclimated rainbow trout), which is approximately

60% that of starved seawater-acclimated rainbow trout (Wilson *et al.*, 1996). It is intriguing to speculate about why an intestinal phenotype that is characteristic of marine teleosts (Wilson, Wilson and Grosell, 2002; Grosell, 2006) should be present in a freshwater fish. Given that the production of intestinal carbonates was greatly stimulated by diets with elevated calcium content, it is possible that the intestinal process of alkaline carbonate precipitation actually evolved for calcium homeostasis following feeding, rather than osmoregulation in a hypersaline environment, which has previously been described as its primary role in marine teleosts (Wilson *et al.*, 2009). However, it cannot be ignored that the rainbow trout is a euryhaline species, so this species already possesses within its genome the blueprint for producing the required cellular machinery to drive intestinal carbonate production. It would therefore be informative to attempt similar experiments using purely stenohaline freshwater fish, before refining conclusions about the origins of this gut process in fish more generally.

Chapter 4: General discussion

The present study adds valuable evidence to the current literature on carbonate production by fish. In chapter one I identified a number of important gaps in our knowledge of marine fish carbonate production. In chapter two I investigated the quantity and composition of carbonates excreted by a particular marine teleost species (lumpfish) for the first time and established the Q_{10} across the lowest temperature range previously quantified in a marine species. In chapter three I investigated how food intake affects carbonate excretion, for only the second time (Newbatt, 2015). This included diets containing calcium salt concentrations ($CaCO_3$ and $Ca_3(PO_4)_2$) representative of calcified invertebrates and bony fish prey, and I characterised intestinal carbonate production and excretion in a freshwater-acclimated teleost for the first time.

The effect of cold temperatures on carbonate production is extremely important to our understanding of global carbonate production by fish, especially with predicted ocean warming associated with climate change (IPCC Panel, 2014). Many species inhabit high latitudes, where seawater from the surface of the ocean to the seafloor is all within this temperature range. Additionally, seawater gradually decreases in temperature below the surface mixed layer. Mesopelagic species such as myctophids (lanternfishes) may make up >90% of global fish biomass, and spend a significant proportion of their time in cold, deep water due to undergoing diel vertical migrations (Watanabe *et al.*, 1999). These fish have been implicated in a proposed upward alkalinity pump, which may act to mitigate atmospheric climate change by transporting alkalinity from deep to surface water, allowing the surface ocean to absorb more CO_2 from the atmosphere (Roberts *et al.*, 2017). I calculated a Q_{10} of 3.50 for carbonate production in lumpfish between 3 and 7 °C, which is comparable to Q_{10} values calculated for temperate and tropical species (Reardon *et al.*, in preparation; Wilson *et al.*, 2009; Heuer *et al.*, 2016). This confirms that fish continue to produce carbonates at temperatures <5 °C, and that the excretion rate bears a similar relationship to temperature at low temperatures, as it does at high temperatures. I found that across the range of temperatures tested, the magnesium content of precipitates remained constant, similarly to many other examined species (Salter, Perry and Smith, 2019). This suggests that the solubility of carbonates is unaffected by the temperature at which they are formed, and any changes in dissolution rate of carbonates in the

environment is more likely related to temperature-associated changes in Ω_{CaCO3} in the external seawater, i.e., post-excretion.

It is dangerous to extrapolate from a single studied species to all teleosts inhabiting cold marine environments. As mentioned in chapters one and two, carbonate excretion rate may not bear a strictly exponential relationship with temperature. Instead, it may resemble the relationship between metabolic rate and temperature demonstrated in Atlantic halibut (*Hippoglossus hippoglossus* - Gräns *et al.*, 2014), with carbonate production being relatively constant across the middle portion of their temperature tolerance range, and more strongly affected towards the upper and lower limits. If this is the case, the part of their temperature tolerance range that a fish population actually inhabits will be important to understanding their response to a warming ocean. Chapter two's data on lumpfish disagrees with data on flounder that suggests magnesium content is strongly affected by temperatures <12 °C (Reardon *et al.*, in preparation). If the example of flounder is the case for most species, it would challenge our assertion that temperature-associated changes in Ω_{CaCO3} would be the primary driver of altered carbonate dissolution rate. However, our data agree well with most studies on the relationship between temperature and carbonate excretion rate (Reardon *et al.*, in preparation; Wilson *et al.*, 2009; Heuer *et al.*, 2016) and magnesium content of carbonates in most species (Salter, Perry and Smith, 2019) at temperate and tropical temperatures. These data from lumpfish are particularly valuable as they provide the first insight into carbonate production within the lower portion of the full range of seawater temperatures (-2 – 36 °C). This means that future modelling efforts can use data derived from cold water species, rather than extrapolating from temperate and tropical species.

The effect of dietary intake on carbonate production represents perhaps the most significant gap in the literature as of the commencement of this project. Fish spend a significant proportion of their lifetime in a fed state, and likely consume a large amount of calcium in the form of $CaCO_3$ (in invertebrate shells or exoskeletons) or $Ca_3(PO_4)_2$ (in bony fish skeletons). However, many studies have investigated carbonate production due to its role in osmoregulation or acid-base balance (Wilson *et al.*, 1996; Wilson and Grosell, 2003; Grosell *et al.*, 2005) and have omitted feeding as this complicates the examination of these processes, due to the intestine performing additional functions. Studies on fish carbonate

production have also usually used starved fish due to the need to convince sceptical ocean carbon scientists (who work on other calcifying species) that the fish carbonates are actually produced de novo in their intestines and are not simply ingested calcareous prey passing through their guts intact. Chapter two's data on carbonate production by freshwater rainbow trout fed diets supplemented with calcium salts suggest that organisms consuming calcium in the form of $CaCO_3$ will excrete more intestinal carbonate mineral than those consuming diets containing calcium in the form of $Ca_3(PO_4)_2$. This is likely to be due to the re-precipitation of $Ca_3(PO_4)_2$ in the intestine after being dissolved in the stomach (creating free Ca^{2+} and PO_4^{3-} ions) and the subsequent inhibition of carbonate formation in the intestine by phosphate ions (Morse, Arvidson and Lüttge, 2007), among other factors. In the marine environment, carbonate excretion rates from fed fish are likely to be even higher than those observed in freshwater fish, as additional calcium is provided in seawater ingested for osmoregulation, and also because seawater drinking rates tend to be higher when feeding than when starved.

A fascinating outcome of the results from chapter three is that intestinal HCO_3^- concentration of fed freshwater-acclimated rainbow trout are very similar to those measured in fed seawater-acclimated rainbow trout (Wilson *et al.*, 1996). This led me to speculate that intestinal HCO_3^- secretion may have evolved for calcium homeostasis following feeding, rather than its suggested primary purpose of osmoregulation in marine fish (Wilson *et al.*, 2009). As all marine fish may be derived from a freshwater ancestor (Carrete Vega and Wiens, 2012), if carbonate excretion evolved for osmoregulation, it would not be present in freshwater species with no history of inhabiting the marine environment. It would therefore be particularly interesting to test whether a stenohaline freshwater species, with no marine ancestry, is capable of excreting carbonates in response to high calcium concentrations in its diet.

Many knowledge gaps exist within the field of teleost carbonate production. To date, only a single study has investigated the solubility or dissolution of piscine carbonates, and only in a single species (Woosley, Millero and Grosell, 2012). No data exists on the sinking rate of carbonates, or their sinking or dissolution rates when excreted with faeces, which is probably representative of most carbonates excreted into the environment. If metabolic rate and carbonate

excretion rate were measured simultaneously in the lab, this could be usefully integrated with the wide range of existing data on metabolic rate measured in the wild. This is important as animal behaviour under experimental conditions is rarely representative of natural behaviour in the wild. As metabolic rate is tightly linked to carbonate production rate (Wilson *et al.*, 2009), this would be helpful to better predict carbonate excretion rate in the wild from measurements of carbonate excretion in lab-based experiments. This is import as it facilitates more accurate global modelling of the inorganic carbon cycle, which has implications for how much CO_2 the ocean can absorb from the atmosphere and therefore mitigate climate change.

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