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The Stress of Subordinance:
Socially Mediated Differences in Acid-Base Regulation in Rainbow Trout
(*Oncorhynchus mykiss*)

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THE STRESS OF SUBORDINANCE: SOCIALLY MEDIATED DIFFERENCES IN ACID-BASE REGULATION IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Abstract

Rainbow trout (*Oncorhynchus mykiss*) held in pairs engage in agonistic interactions resulting in one fish becoming dominant over the other, subordinate fish. Subordinate social status constitutes a chronic stress with subordinate fish exhibiting a prolonged elevation of circulating cortisol levels that in turn leads to reduced growth rates, altered metabolic function and compromised immune responses. The chronic stress experienced by subordinate fish also impacts ionoregulatory ability. Owing to the tight coupling between Na^+ and Cl^- uptake and, respectively, H^+ and HCO_3^- excretion at the gill, such ionoregulatory changes may affect acid-base regulation. Thus, the present study investigated the impact of social status on acid-base regulation of respiratory acidosis in rainbow trout. Responses of subordinate, dominant and control trout to 24 h of hypercapnia were compared.

Social status appeared to impact net acid excretion ($J_{net}H^+$) as subordinate individuals were unable to increase net acid flux in response to hypercapnia. However, despite this impaired response, blood acid-base status was found to be unaffected by social status before or during hypercapnic exposure, indicating that subordinate fish were as effective as dominant or control trout in achieving compensation for the acid-base disturbance. Compensation in all groups involved decreasing Cl^- uptake in response to hypercapnia, thereby reducing HCO_3^- loss. Differences in branchial and renal Na^+, K^+-ATPase and H^+-ATPase activities were documented between subordinate and dominant/control trout. Subordinate individuals exhibited elevated circulating cortisol concentrations prior to hypercapnia and failed to exhibit further increases in circulating cortisol levels in response to the acute stress of hypercapnia. Taken as a whole, the findings of this study suggest that subordinate fish are able to regulate body fluid pH during a respiratory acidosis, but this regulation may come at a greater cost than is the case in dominant or control fish.

Résumé

La truite arc-en-ciel (*Oncorhynchus mykiss*) se regroupant en pair occasionne une interaction agonistique résultant un statut de dominance d'un poisson envers un autre poisson subordonné. L'interaction sociale constitue un stress chronique chez les poissons subordonnés avec une augmentation prolongée du niveau de cortisole, qui en retour entraîne une réduction du taux de croissance, modifie les fonctions metaboliques et compromet la réponse immunitaire. Le stress chronique subit par le sujet subordonné a aussi un impact sur l'abilité de régulation des ions, ayant pour conséquence la dépendance serrée entre la consommation de Na^+ et Cl^- et, l'élimination de H^+ et HCO_3^- au niveau des branchies. Ce type de régulation peut en retour affecter la régulation acide-base. Ainsi, cette étude se concentre sur l'impact du statut social sur la régulation acide-base de la respiration acidique chez la truite arc-en-ciel (*Oncorhynchus mykiss*), où trois groupes expérimentaux ont été examinés (subordonné, dominant et contrôle) pendant 24 h en condition d'hypercapnie.

Le statut social semble avoir un impact sur le flux net d'acide ($J_{net}H^+$) pendant que les sujets subordonnés étaient incapables d'augmenter le flux net d'acide en condition d'hypercapnie. Malgre cette incapacité, le status acide-base dans le sang se retrouve être moins affecté par le status social avant et pendant l'exposition en condition d'hypercapnie, indiquant que les poissons subordonnés, aussi bien que les groupes expérimentaux (dominant et contrôle), semblent compenser pour la condition acide-base, ainsi empêchant la perte en HCO_3^-. Les différences dans l'activité branchiale et renale de Na^+,K^+-ATPase et H^+-ATPase ont été observées chez les truites subordonnés, dominants et contrôles. Les individus subordonnés démontrent une concentration élevé en cortisol sanguin avant l'hypercapnie et n'ont pas démontré une augmentation de ce dernier durant l'exposition prolongée autre stress due à l'hypercapnie. En tout, les résultats obtenus suggèrent que les poissons subordonnés

sont capables de faire la régulation du pH du fluide corporel durant la respiration acidique, cependant cette régulation pourrait être plus exigente (demandant) comparé aux sujets dominants et contrôles.

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Abbreviations

CA, carbonic anhydrase

GR, Glucocorticoid receptor

Hb, haemoglobin

HPI, hypothalamo-pituitary-interrenal axis

HSP, heat shock proteins

$J_{net}H^+$, net acid flux

$J_{net}TA$, net titratable alkalinity

$J_{net}NH_3$, net ammonia flux

MCHC, Mean corpuscular haemoglobin concentration

MR, mineralocorticoid receptor

MR cell, mitochondrion rich cell

NBC1, Na^+,HCO_3^- co-transporter

NEM, N-ethymaleimide

NHE, Na^+/H^+ exchanger

NKA, Na^+,K^+-ATPase

NKCC, Na^+,K^+, 2 Cl^- co-transporter

PCO_2, partial pressure of carbon dioxide

$PaCO_2$, partial pressure of carbon dioxide in arterial blood

pHa, arterial pH

PNA, peanut lectin agglutinin

PV cell, pavement cells

RBC, red blood cell

sem, standard error of the mean

Introduction

The impact of environmental changes on the physiology of has long been of interest to comparative physiologists, but such studies have largely focused upon physico-chemical parameters such as temperature, pH, O_2 levels or ion levels. More recently, physiologists have started to take into consideration the biotic environment, including interspecific interactions (e.g. predator-prey relationships) and intra-specific interactions (e.g. social structure). While the interplay between physiology and behaviour within an individual animal has been recognized from some time (Cabanac, 1996), comparative physiologists have more recently placed greater emphasis upon this perspective (e.g. Gilmour et al., 2005b). Comparative physiology has expanded to adopt a more integrative approach, including consideration, and experimental manipulation of the intimate link between behaviour and physiology. The phenomenon of social hierarchy formation is a prime example of the impact of behaviour, or the social environment, on physiological condition or responses. First documented in chickens (Schjelderup-Ebbe, 1922), pecking order structures have since been described in a variety of vertebrates, including fish (Huntingford and Turner, 1987). Social hierarchy formation is generally driven by competition for limited resources such as food, shelter, and mates (Chapman, 1966). While social hierarchy formation occurs in many species of teleost fish, driven by any or all of these factors (Scott and Curie, 1980; Koebele, 1985; Fox et al., 1997), in juvenile salmonids, the focus of the present work, competition is driven by limited food resources and the benefit of shelter (Bachman, 1984; Metcalfe et al., 1989; Huntingford, 1997).

The existence of social hierarchies implies individual differences in behaviour and competitive ability, which in turn suggest the existence of innate differences in physiology. Moreover, differences in social status can have significant physiological consequences. For

example, social status can have a profound effect on future life history by influencing the time at which salmon metamorphose into smolts and undertake the seaward migration (Metcalfe et al., 1989; Metcalfe et al., 1990; Metcalfe and Thorpe, 1992). The influence of social status on life history variation depends on the competitive ability of a juvenile salmonid; by out-competing other individuals a dominant salmonid acquires the necessary food to build the reserves required to undertake migration and thus initial size is a consequence of social status (Metcalfe and Thorpe, 1992). Consideration of differences in physiology and behaviour can also be of economic benefit. For example, in aquaculture a better understanding of the consequences of physiology-behaviour interactions can lead to improved methods of rearing and maintaining large groups of fish (Thorpe and Cho, 1995).

With this broad background in mind, the goal of the present study was to investigate the physiological consequences of social status in rainbow trout, with a specific focus on acid-base regulation. Rainbow trout, *Oncorhynchus mykiss*, are a useful model for this work not only because consistent stocks can be purchased from commercial fish farms, but also because social hierarchy formation readily occurs in this species both under natural conditions and in artificial settings (Bachman, 1984; Sloman and Armstrong, 2002). In addition, many of the direct physiological consequences of social status in rainbow trout have been documented (Gilmour et al., 2005), providing a framework within which to examine potential downstream or secondary effects of social status. In the remainder of this Introduction, social hierarchy formation in juvenile salmonids will be briefly reviewed together with known physiological consequences of social status. Acid-base regulation in freshwater fish will also be reviewed, leading to the generation of specific hypotheses and predictions concerning the possible impact of social status on the response of rainbow trout to hypercapnia.

Social stress

The phenomenon of social hierarchy formation has been well studied in salmonids (e.g. Bachman, 1984; Metcalfe et al. 1989; Huntingford and de Leaniz, 1997; Gilmour et al., 2005a). Small groups of juvenile rainbow trout readily form linear dominance or pecking order hierarchies in the wild and in laboratory settings; these hierarchies are established through aggressive interactions (reviewed by Sloman and Armstrong, 2002). The formation of social hierarchies among juvenile salmonids is driven by the need to gain access to limited resources within the environment (Kalleberg, 1958; Noakes and Leatherland, 1977; Bachman, 1984; Nakano, 1995; Adams et al., 1998). Specifically, juvenile salmonids in their natal streams compete for feeding territories. Therefore, higher-ranking fish secure a feeding advantage over lower-ranking fish, and this difference influences their future life history (Metcalfe et al., 1989). Juvenile fish that fail to establish a feeding territory are forced to emigrate and may experience increased risks of mortality (Elliot, 1994). The competitive imperative is such that pairs of juvenile rainbow trout confined together in an otherwise bare aquarium will engage in agonistic interactions to establish dominance (Sloman et al., 2000b; Gilmour et al., 2005a).

The behaviours of dominant and subordinate individuals are well documented (reviewed by Winberg and Nilsson, 1993). By out-competing other fish in agonistic encounters, dominant individuals obtain the better positions within the environment and a larger share of the available food while exhibiting greater levels of aggression and activity (Fausch, 1984; Abbott and Dill, 1985; Abbott et al., 1985; Metcalfe, 1986; Metcalfe et al., 1989; Huntingford et al., 1990; McCarthy et al., 1992; Sloman et al., 2000b). These individuals also tend to monopolize essential resources, forcing subordinate fish to be

excluded from these resources and potentially to suffer higher mortality (McCarthy et al., 1992; Elliott, 1994; Kadri et al., 1996). By contrast, subordinate individuals exhibit marked behavioural inhibition, including reduced activity, feeding and aggression (Abbott and Dill, 1985; McCarthy et al., 1992; Winberg et al., 1993b; Moutou et al., 1998; Overli et al., 1998). Many of these behavioural correlates of social status are thought to reflect changes in brain monoaminergic activity that occur as a consequence of either winning or losing agonistic social encounters (Winberg and Nilsson, 1993). For example, an elevation in serotonergic activity in subordinate salmonid fish compared to dominant individuals (Winberg et al., 1992; Winberg and Nilsson, 1993; Winberg and Lepage, 1998; Overli, 1998) has been linked to the suppression of feeding (Overli, 1998), activity (Winberg et al., 1993b) and aggression (Winberg et al., 2001). The intimate link between physiology and behaviour is clear from the demonstration of brain monoaminergic activity as both a physiological mediator of changes in behaviour and a physiological response to behavioural interactions (reviewed by Gilmour, 2005).

Individual differences in behaviour and competitive ability are the driving force for the existence of social hierarchies. Several studies have attempted to identify the behavioural and/or physiological characteristics that predict social status. Studies by Metcalfe et al. (1995), Yamamoto et al. (1998) and McCarthy (2001) on Atlantic salmon and Masu salmon, and rainbow trout, respectively, found dominance correlated with standard metabolic rate and not body mass (reviewed in Sloman and Armstrong, 2002). High metabolic rate may result in high metabolic scope (the magnitude through which the aerobic metabolic rate can vary from standard metabolic rate to maximum metabolic rate) allowing for greater levels of aggression (Priede, 1985) or the increased metabolic demands may drive aggressive behaviour in response to hunger (Metcalfe et al., 1995). Other predictors of

future social status include competitive ability and prior experience of an individual fish (Abbott et al., 1985; Huntingford and de Leaniz, 1997; Rhodes and Quinn, 1998; Johnsson et al., 1999; Cutts et al., 1999a, 1999b). How fish assess competitive ability is not well understood. However, in some salmonids it appears size differences can indicate greater competitive ability (Abbott et al., 1985). An individual that is subordinate in one social encounter is more likely to remain subordinate in subsequent encounters (Abbott et al., 1985; Rhodes and Quinn, 1998; Johnsson et al., 1999), possibly due to the changes in brain monoaminergic activity that reinforce submissive characteristics (Winberg et al., 1993). Evidence also suggests that high levels of circulating cortisol can predict low social status in subsequent pairings (Sloman et al., 2001; DiBattista et al. 2004). Although some indicators of potential social status are available from such studies, the mechanistic links between these parameters and future social status are not well understood (reviewed by Sloman and Armstrong, 2002).

Stress of subordinance

Although both dominant and subordinate fish benefit from hierarchy formation, as energy expended on aggression declines once the hierarchy is formed (reviewed by Sloman and Armstrong, 2002), there are persistent detrimental consequences for subordinate individuals (Abbott and Dill, 1989; Pottinger and Pickering, 1992; reviewed by Sloman and Armstrong, 2002; Gilmour et al., 2005). One of the most obvious consequences of subordinance is a reduction in growth rate (Abbott and Dill, 1989; Metcalfe et al., 1990; Pottinger and Pickering, 1992), an effect that is in part due to the monopolization of food sources by dominant individuals (Metcalfe et al., 1989; McCarthy et al., 1992; Winberg and Nilsson, 1993a; Adams et al., 1998; Maclean and Metcalfe, 2001). However, even when

dominant and subordinate fish are fed separately under circumstances where subordinates are not in contact with dominant individuals during feeding, subordinates may exhibit anorexia (DiBattista et al., 2006). Additionally, lower growth rates in subordinates are still observed when both members of a pair consume equal rations (Abbott and Dill, 1989), suggesting that low growth rates also reflect additional factors attributed to subordinance apart from food monopolization (reviewed by Sloman and Armstrong, 2002).

The differences in growth rate among social statuses of salmonids may be explained by the prolonged elevation of circulating cortisol levels detected in subordinate fish, a second very well-characterized consequence of social interactions (Overli et al. 1999a; Sloman et al. 1999a; Pottinger and Pickering, 2002; reviewed by Sloman and Armstrong, 2002; Gilmour et al., 2005). Immediately following the initiation of social interactions, dominant and subordinate individuals both exhibit a large increase in plasma cortisol (Overli et al., 1999b; Sloman et al., 2001a). As aggressive interactions abate with hierarchy formation (within ~3 h), dominant fish exhibit a decrease in cortisol so that circulating levels become comparable to those of unstressed controls, while subordinates continue to show elevated cortisol concentrations for up to a week of social interaction (Overli et al., 1999b; Sloman et al., 2001a). Cortisol concentrations appear to be correlated with behavioural interactions, where higher circulating levels of cortisol prior to social interactions are associated with more submissive behaviour (Sloman et al., 2001a). Following hierarchy formation, chronic elevation of cortisol in subordinates appears to impair activation of the hypothalamo-pituitary-interrenal (HPI) axis in response to additional stressors, while dominant individuals remain capable of responding appropriately to an acute stressor by activation of the HPI axis (Overli et al., 1999b; Sloman et al., 2002).

Subordinate fish exhibit physiological changes that result from the maintained elevation of this stress hormone. Cortisol is a corticosteriod hormone that is synthesized and released from interrenal cells in the anterior (head) kidney of teleost fish as the main steroid stress response hormone (reviewed in Wendelaar Bonga, 1997). The activation of stress hormone pathways mediates appropriate responses to stressors that are required to regain or maintain the normal or homeostatic state (Barton, 2002). Cortisol mediates widespread changes including changes in plasma and tissue ion and metabolite levels, haematological parameters, and heat-shock or stress proteins (HSPs), which result in physiological adjustments in metabolism, respiration, immune function and cellular responses (Pickering and Christie, 1981; Iwama et al., 1998; Mommsen et al., 1999). Long-lasting stressors lead to prolonged elevation of circulating cortisol which can be detrimental to the health and well being of the fish. Physiological changes observed in subordinate fish as a result of chronically elevated cortisol concentrations are thought to include an increase in metabolic rate (Sloman et al., 2000c), mobilization of energy reserves (Ejike and Schreck, 1980; Sloman et al., 2001a; reviewed by Gilmour et al., 2005), alterations in metabolic function (DiBattista et al., 2006) and depression of digestive function (Horn, 1998; Earley et al., 2004; DiBattista et al., 2006). All of these can help to explain the decline in growth rate observed even when subordinate fish are fed in isolation (Abbott and Dill, 1989). The chronic elevation of circulating cortisol also results in an overall poor condition of subordinate fish (reviewed in Gilmour et al., 2005) because chronic elevation of cortisol depresses immune function. When combined with their overall poor condition, subordinate fish become susceptible to bacterial and/or fungal infections; although the extent to which this occurs in natural environments is unknown (Abbott et al., 1985; Moutou et al., 1998; Pottinger and Pickering, 1992). In addition to the role of cortisol in metabolism and immune function, it is

also thought to have an effect on ionic/osmotic regulation in fish by altering the gill epithelia at both the cellular and molecular levels (Laurent and Perry, 1990; Perry and Laurent, 1992) and therefore, the chronically elevated plasma cortisol concentrations seen in subordinate fish may impact upon ionic and/or osmotic regulation. Subordinate fish exhibit significantly higher Na^+ uptake rates than dominant fish (Sloman et al., 2003b, 2004). This difference is probably driven by the need to correct for higher branchial Na^+ efflux rates in subordinate fish, to maintain Na^+ balance (Sloman et al., 2004). The altered rates of sodium movement in subordinate trout appear to have downstream consequences, as subordinate fish show greater susceptibility to environmental toxins such as copper, cadmium and silver (Sloman et al., 2002a, 2003b, c). Sloman and colleagues (2002a, 2003b, 2004) determined that the greater uptake of Na^+ in subordinate fish is responsible for the socially-mediated differences in copper uptake because copper is thought to enter fish via a sodium channel, or via another Na^+ uptake mechanism (Grosell and Wood, 2002). It is noteworthy that Sloman et al. (2000) failed to find any impact of social status on the prevalence of ion-transporting mitochondrion-rich (MR) cells in the rainbow trout gill epithelium. However, changes in branchial epithelium cell composition are but one effect of many that appear to be influenced by circulating cortisol concentrations.

Much of the previous work on hierarchy formation in salmonid fish has centered on understanding the specific behaviours associated with hierarchy formation (e.g. Sloman and Armstrong, 2002; Gilmour et al., 2005), as well as the physiological traits associated with the different social ranks and the physiological changes mediated by social status (e.g. Winberg and Nilsson, 1993; Sloman and Armstrong, 2002; Gilmour et al., 2005). However, less is known of the impact of social status on the ability of fish to cope with environmental challenges. The findings of Sloman and colleagues (2002a, 2003b, c) on differences in Na^+

regulation resulting from social status and related impacts on susceptibility to toxic metals in the environment has led to questions of the potential impact a chronic stress such as social interaction can have on ionic and therefore acid-base regulation. In fish, acid-base regulation and ionic regulation are tightly linked (see below). Thus, changes in ionic regulation resulting from social stress may impact upon acid-base regulation. A widely applied laboratory method used to characterize acid-base regulation is exposure to the acid-base challenge of external hypercapnia (elevated CO_2 tension in water) (Cameron, 1978). Exposure to hypercapnia elevates internal CO_2 tension and consequently lowers pH, causing a respiratory acidosis. Compensation of this respiratory acidosis requires dynamic changes in the systems that underlie ionic regulation and acid-base regulation.

Acid-base regulation in freshwater fish

Freshwater fish live in an environment that is hypo-osmotic to their body fluids and must cope in consequence with water entry and salt loss across permeable membranes (e.g. the gills). Salt and water balance are achieved through active ion uptake across the gills coupled with water excretion via the kidney (reviewed by Evans et al., 2005). Freshwater fish must also cope with a dynamic environment where changes in pH and/or ion levels are common occurrences. Regulation of salt, water and acid-base balances all rely largely upon the direct exchange of ions between the fish and its environment which occurs across the gills (Goss et al., 1992a; Claiborne et al., 2002; reviewed in Evans et al., 2005). Unlike air-breathing vertebrates, fish make little use of changes in ventilation rate to regulate acid-base disturbances (reviewed by Perry and Gilmour 2006). Through the reversible hydration/dehydration reaction of CO_2 and the acid-base equivalents H^+ and HCO_3^- catalyzed by the enzyme carbonic anhydrase (CA) ($CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$), acid-base regulation

is intimately linked to changes in CO_2 levels (Perry and Laurent, 1990; reviewed by Perry et al., 2003b). Thus, changes in breathing, or respiratory compensation, can be used to regulate acid-base balance. However, this approach is not feasible for fish as reductions in breathing to compensate for an alkalosis (high pH) negatively impact O_2 delivery, while the capacity to lower CO_2 levels in body fluids by increasing ventilation to compensate for an acidosis (low pH), is limited by the very low CO_2 levels exhibited by water-breathing fish (Claiborne et al., 2002; Perry and Gilmour 2006). The more useful mechanism for water-breathing fish to offset changes in pH is via direct transepithelial transfer of acid-base equivalents between the animal and the external environment (metabolic compensation). Metabolic compensation is achieved by adjusting the transfer of acidic (H^+) and basic (HCO_3^-) equivalents between the internal and external environments (reviewed by Perry and Gilmour, 2006). Compensation for an acidosis involves H^+ loss and/or HCO_3^- gain, whereas H^+ accumulation and/or HCO_3^- loss are used to compensate for an alkalosis. Metabolic compensation is accomplished by excretion of H^+ and HCO_3^- in exchange for the uptake of Na^+ and Cl^- ions, respectively (Claiborne et al., 2002; Perry et al., 2003b; Evans et al., 2005). Therefore, acid-base regulation is intimately linked to ionic regulation since acid-base equivalents must be excreted to take up Na^+ and Cl^- from the water. Moreover, adjustments of acid-base balance may impact upon ionic regulation. Although the transport mechanisms in many cases have yet to be identified at the molecular level, it is widely accepted that the excretion of H^+ is linked to the uptake of Na^+, while the excretion of HCO_3^- is linked to Cl^- uptake (reviewed by Perry and Gilmour, 2006).

Ionic and acid-base regulation in fish is carried out at the gills and kidney (reviewed in Evans et al., 2005; Perry et al., 2003b). The critical importance of the gills in achieving acid-base compensation is widely accepted; the gills are thought to account for

approximately 90% of acid-base movements (Claiborne et al., 2002). Sodium ion uptake in exchange for protons is thought to occur through an apical Na^+ channel coupled to an apical H^+-ATPase, with Na^+ exiting the gill epithelium at the basolateral surface through Na^+/K^+-ATPase (Fenwick et al., 1999; reviewed in Evans et al., 2005). Chloride (Cl^-) ions are thought to be exchanged for bicarbonate (HCO_3^-) ions at the apical surface and transferred across the basolateral membrane via a Cl^- channel down the electrical gradient (reviewed by Goss et al., 1992, 1995; McCormick, 2001; Evans et al., 2005; Hwang and Lee, 2007). The sites and mechanisms of acid-base equivalent transfer in freshwater fish gills are not fully understood, but appear to involve different types of MR cells in the gill epithelium (Perry and Gilmour 2006). The gill epithelial cells involved in acid-base regulation are the MR cells (also sometimes called chloride cells or ionocytes) and the pavement cells (PV cells; also called respiratory cells). In rainbow trout, MR cells are further classified by morphological differences; those exhibiting peanut lectin agglutinin (PNA) binding sites on the apical membrane (PNA^+ MRCs) and those lacking PNA binding sites (PNA^- MRCs) (Goss et al., 2001; Galvez et al., 2002).

The kidney plays a key supporting role in acid-base regulation by allowing changes at the gills to be maintained (Wood et al., 1999; Georgalis et al., 2006a; see reviews by Perry and Fryer, 1997; Perry et al., 2003b). Without retention at the kidney of accumulated HCO_3^- at the kidney, branchial acid excretion becomes a wasted effort. During systemic acidosis, renal acid excretion is increased mainly in the form of proton and inorganic phosphate excretion (Wood et al., 1999). Under resting conditions, renal HCO_3^- reabsorption relies on hydration of CO_2 catalyzed by cytosolic CA and the subsequent extrusion of H^+ into the filtrate of the proximal tubule where it combines with filtered HCO_3^- in a reaction catalyzed by luminal CA IV to form CO_2 that moves back into the renal tubule cell. Hydration of this

CO_2 yields HCO_3^- ions that exit the cell basolaterally, resulting in net HCO_3^- transfer from filtrate to blood (Perry et al., 1987b). This basic mechanism is very similar to that in mammals (Romero and Boron, 1999; Swenson, 2000). During an acid-base disturbance such as hypercapnia, acid-base regulation at the gills results in increased branchial acid output and accumulation of HCO_3^- in the plasma (Wood and Jackson, 1980; Wheatly et al., 1984; Perry et al., 1987b) a process the is supported at the kidney by increasing acid secretion and HCO_3^- reabsorption. In particular, enhanced reabsorption of HCO_3^- ions from the renal filtrate is essential for HCO_3^- accumulation to compensate for acidosis. Enhanced HCO_3^- reabsorption is achieved at least in part by increased expression and protein levels of both cytosolic and membrane bound CA isoforms in the proximal tubule (Georgalis et al., 2006b).

Hypercapnia

The tight linkage between acid-base regulation and ionic regulation via ion transport proteins in the gills and kidney means that any changes in one parameter (i.e. acid-base or ion balance) will be reflected in the other (Claiborne et al., 2002; Perry et al., 2003a,b). Hypercapnia is an invaluable tool for the study of reactions to disturbance of the acid-base state because small increases in ambient water CO_2 levels produce large changes in blood pH (Cameron, 1978). During hypercapnia, fish are exposed to an environment that results in CO_2 accumulation within the body fluids, and this accumulation in turn decreases body fluid pH owing to the equilibrium $CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$. To regulate internal pH, fish accumulate HCO_3^- ions so as to achieve a new equilibrium in which pH is brought back to the control value while PCO_2 and HCO_3^- remain elevated (Cameron and Randall, 1972; Janssen and Randall, 1975; Cameron, 1978). The most significant response to combat

respiratory acidosis induced by hypercapnic exposure in rainbow trout is through the inhibition of branchial chloride uptake; lowering branchial Cl^- uptake results in a subsequent accumulation of bicarbonate ions (reviewed by Heisler, 1984; Perry et al., 1987a). Fish may simultaneously increase branchial acid excretion (Wood et al., 1984; Perry et al., 1987a), and species-specific differences exist in the extent to which these two mechanisms are employed. Within rainbow trout, inhibition of Cl^- uptake appears to trump enhanced branchial H^+ excretion (Perry et al., 1987a). Although the gill is the major site of acid-base regulation (Cameron, 1978), complementary changes in renal acid secretion are required to retain the accumulated HCO_3^- (Perry et al., 1987b). Recent work indicates that renal compensation is important for retaining the accumulated bicarbonate by increasing acid secretion into the filtrate to promote reabsorption of filtered HCO_3^- (Georgalis et al., 2006b). Trout CA IV is preferentially expressed in the posterior kidney and mRNA levels become elevated following 24 h of hypercapnia exposure (Georgalis et al., 2006b). This CA isoform (and potentially others) play an important role in acidification of the urine and renal HCO_3^- reabsorption (Georgalis, 2006b).

Control systems: the importance of cortisol

The process of detecting and responding to changes within the external environment requires the involvement of the neuroendocrine system. The neuroendocrine system is thought to be the primary link between environmental changes and the appropriate physiological response mediating its effects through hormones (Wendelaar Bonga, 1997; Mommsen et al., 1999; McCormick, 2001). Previous studies have shown that a number of hormones, including prolactin, growth hormone and cortisol, are involved in osmoregulation (reviewed by McCormick, 2001). However, cortisol is of particular interest in the current

work owing to the manner in which it is affected (as a stress hormone) by social status (see above).

Cortisol has a broad range of action in fish with important targets including the gills, intestine, kidney and liver. These targets reflect the two major regulatory actions of cortisol in fish – cortisol is involved in regulation of hydromineral balance and metabolic function (reviewed in Wendelaar Bonga, 1997; Mommsen et al., 1999). In rainbow trout, cortisol is released into the bloodstream via the HPI axis (reviewed by Wendelaar Bonga, 1997; Mommsen et al., 1999). The stimulation of cortisol release begins with the activation of hypothalamic nuclei to secrete corticotropic releasing factor (CRF) into the anterior pituitary thereby stimulating the release of adrenocorticotropic hormone (ACTH) into the circulation. ACTH then acts on the interrenal cells in the head kidney to elicit the synthesis of cortisol and its secretion into the bloodstream (Balm et al., 1994).

In fish, cortisol acts as both a glucocorticoid and a mineralocorticoid hormone binding and activating both types of corticosteroid receptors (GR, glucocorticoid receptor, and MR, mineralocorticoid receptor) (Prunet et al., 2006; Bury and Sturm 2007). Interestingly, these receptors are named for their mammalian counterparts based on gene sequence similarity. Trout have two forms of GR, GR1 and GR2, which are known to mediate both metabolic and iono/osmoregulatory events (Bury et al., 2003) but the function(s) of the MR remain unclear at present (Sturm et al., 2005). With respect to hydromineral balance, for example, cortisol has stimulatory effects on branchial Na^+ and Cl^- uptake (Laurent & Perry, 1989), as well as the ability to promote differentiation of ion-transporting MR cells (reviewed in McCormick, 1995; Wendelaar Bonga, 1997). Cortisol was traditionally thought to be the "seawater-adapting hormone", but recent work suggests that it also plays a role in acclimation to dilute environments (McCormick, 2001; Sloman et

al., 2001). Cortisol is implicated in causing increases in branchial, renal, and intestinal Na^+,K^+ -ATPase (NKA) activity (Laurent and Perry, 1990; McCormick, 1995; Takahashi et al., 2005). Cortisol also increases the number of MR cells in several teleosts, such as rainbow trout (*Oncorhynchus mykiss*), Coho salmon (*Oncorhynchus kisutch*), and the American eel (*Anguilla rostrata*) (Madsen, 1990; McCormick, 1995). Cortisol mediates effects on metabolic function by influencing carbohydrate, protein, and lipid metabolism. For example, cortisol has been reported to alter hepatic glycogen levels, as well as to increase hepatic glucose (Vijayan et al., 1991; Randall & Perry, 1992).

Goals and Hypothesis

The objective of this study was to understand how social status impacts the ability of rainbow trout to cope with an acid-base challenge. As observed in recent studies (Sloman et al., 2003b, 2004), subordinate trout demonstrated a significantly higher copper uptake and tissue burden over dominant counterparts. This phenomenon was linked to elevated sodium uptake by subordinates, a response not observed in dominants (Sloman et al., 2004). Subordinate fish demonstrated greater sodium efflux which necessitated a compensatory increase in influx. This thesis examines the hypothesis that a similar difference between dominant and subordinate fish will occur with respect to the regulation of Cl^- ions, i.e. that subordinate fish will exhibit significantly higher Cl^- efflux rates and consequently, significantly higher Cl^- uptake rates will be required to maintain Cl^- balance. To maintain significantly higher ion uptake rates, greater branchial Na^+,K^+-ATPase activity is expected in subordinate fish.

Typically, trout increase acid loss (increasing proton excretion; minor component of the response) and retain bicarbonate (which minimizes chloride uptake) to deal with the acid

load incurred by exposure to high CO_2 levels (reviewed by Perry and Gilmour, 2006). If subordinate fish must maintain higher Cl^- uptake rates for purposes of ionic balance, then the ability of subordinate fish to respond to hypercapnia by reducing Cl^- uptake to promote HCO_3^- accumulation is predicted to be impaired. This impairment of metabolic compensation might be apparent in lower rates of net acid excretion during hypercapnia, in reduced HCO_3^- ion accumulation in the plasma, and/or in an attenuation of the lowering of Cl^- uptake during hypercapnia. Finally, the stress of subordinance typically results in elevated circulating cortisol levels in subordinate trout. In response to the additional, acute stress of external hypercapnia, which in rainbow trout not subjected to social interactions elicits a cortisol response (Ivanis et al., 2008b), subordinate trout are predicted to be unable to further increase circulating cortisol levels compared to dominant fish.

To investigate the impact of social status upon acid-base regulation in rainbow trout, three groups of fish were examined, subordinate, dominant and control rainbow trout. Control trout were taken from a large holding tank where conditions are such that social hierarchy formation is expected to be minimal, and are therefore used as a control for the stress of social interaction itself. Trout were exposed to external hypercapnia for 24 h to characterize the acid-base regulatory response and document any differences due to social status. In *series I*, flux experiments under normocapnia and hypercapnia (water PCO_2 ~ 7.6 Torr) were used to determine net acid flux ($J_{net}H^+$). Previous work characterized the movement of Na^+ ions in both subordinate and dominant individuals (Sloman et al., 2003); *series II* collected similar data for chloride (Cl^-) and determined whether any changes in chloride movement occurred with exposure to hypercapnia. *Series III* characterized blood acid-base status during normocapnia and hypercapnia. Finally, *series IV* profiled changes in circulating cortisol with respect to hypercapnia and social status.

Materials and Methods

Experimental animals

Rainbow trout (*Oncorhynchus mykiss*; mass 238 ± 5 g, $N = 110$) were obtained from Linwood Acres Trout Farm (Campbellcroft, ON). Fish were kept on a 12h:12h L:D photoperiod in large fibreglass holding tanks supplied with flowing, aerated and dechloraminated city of Ottawa tap water at 13°C. Fish were allowed to acclimate to holding conditions for at least 2 weeks prior to experimentation. Trout were fed to satiation on commercial trout pellets every second day.

Four experimental series were carried out to investigate differences in acid-base regulation under hypercapnia (elevated water CO_2) in relation to social status. All experimental series compared the responses of "control" fish sampled directly from a holding tank to those of pairs of fish that were held together for 5 days (45 L tank) to allow a social hierarchy to form; within each pair, fish were classified as "dominant" or "subordinate" on the basis of their behaviour (see below). Control fish were held in groups of sufficient size (9-12 fish per 90 L tank) to avoid social hierarchy formation and were fasted for 5 days prior to experimentation to match the limited feeding regime used for behavioural observations. *Series I* examined acid-base regulation by assessing net acid flux ($J_{net}H^+$) through measurements of net titratable alkalinity ($J_{net}TA$) and ammonia flux ($J_{net}NH_3$). In s*eries II*, unidirectional chloride uptake rates were measured radioisotopically and chloride net flux ($J_{net}Cl^-$) was also determined, using a colorimetric method (modified from Zall et al., 1956). In s*eries III*, trout were fitted with a dorsal aortic cannula to examine the acid-base status of arterial blood. Blood samples were used to determine arterial pH, the partial pressure of CO_2 (PCO_2), and HCO_3^- concentration ($[HCO_3^-]$). In s*eries IV*, a profile of the stress hormone (cortisol) response in relation to hypercapnia and social status was

determined. In all experiments, sampling order was alternated among the different groups of fish to control for sequential sampling effects.

To fit trout with a dorsal aortic cannula, fish were anaesthetized by immersion in an oxygenated solution of benzocaine (ethyl-p-aminobenzoate; 0.1 g L^{-1}), weighed, and transferred to a table that provided continuous irrigation of the gills with oxygenated anaesthetic solution. A cannula of flexible polyethylene tubing (PE50; Clay-Adams) was placed in the dorsal aorta according to the method of Soivio et al. (1975). Fish were revived on the surgical table by irrigation of the gills with anaesthetic-free, aerated water and were placed in individual experimental chambers supplied with flowing, aerated water to recover for 24 h. Cannulae were flushed with heparinised (100 IU mL^{-1} ammonium heparin; Sigma) modified (4.5 mmol L^{-1} $NaHCO_3$) Cortland saline (Wolf, 1963). In a previous study (Thomas and Gilmour, unpublished observations), rainbow trout were found to retain their dominant or subordinate social status following implantation of a dorsal aortic catheter. Control fish used in subsequent experiments were held in large groups (9-12 fish per tank; 90 l) and consequently lacked the stress of social interaction.

Behavioural analysis

Social hierarchies were formed within pairs of size matched rainbow trout. Fish were lightly anaesthetized (to the point of losing equilibrium) in a solution of ethyl-*p*-aminobenzoate (0.065 g L^{-1}). Fork lengths (measurement from the tip of the snout to the fork of the tail) were measured and fish were paired according to fork length with no more than a 5% difference (i.e. no more than a 0.5 or 1.0 cm difference; dominants 27.3 ± 0.2 cm

and subordinates 26.9 ± 0.2 cm). Pairs were confined in ~45 L sections of a 90 L fibreglass tank; the tank was separated into sections by a perforated opaque Plexiglas™ divider.

Social status was determined using a variation of the points system that has been used in a variety of studies (e.g. Metcalfe et al., 1989; Johnsson et al., 1996; Sloman et al., 2000a, 2000c). In this scoring system, more dominant behaviours are given more point. Pairs were observed for five days twice daily for 10 min during which the position of each fish and the number of aggressive attacks it carried out were noted. For position observations, fish patrolling the middle of the water column received a score of 10, those at the bottom of the tank or hiding behind a shelter were given a score of 5, and those at the surface of the water column were given a score of 0. Aggressive attacks consisted of nips or bites directed at the other fish and were scored as follows: 0 for no attacks, 1 point for 1-5 attacks, and 2 points for >5 attacks. On the second day of pairing, one food pellet was dropped into the tank to determine readiness to feed, and the fish that first attempted to consume or consumed the food received a score of 1. The feeding regime was adjusted from previous work to minimize impact of food elimination on subsequent experiments.

The final factor used to determine social status was fin damage. Aggressive attacks are generally directed towards the caudal and dorsal fins (Moutou et al., 1998; Turnbull et al., 1998). Therefore, fin damage was used as another measure of social status by comparing damage prior to pairing (only fish with minimal fin damage prior to pairing were used) with that present at the end of the interaction period. Both caudal and dorsal fins were assessed for damage as absent (3 points), minor (<30% of fin missing; 2 points), moderate (30-70% of fin missing; 1 point), or severe (>70% of fin missing, 0 points). This method of assessing fin damage has been used previously (Moutou et al., 1998).

A single behavioural score was then calculated from the mean scores over all observation periods for each fish for tank position, aggression, feeding, and fin damage using principle components analysis (SPSS 15.0; SPSS Scientific Inc., Chicago, IL, USA). Within each pair, the fish with the higher behaviour score was assigned dominant social status, whereas the fish with the lower score was deemed to be the subordinate. Any pair with a behaviour score difference of less than 0.5 was eliminated from subsequent experiments (1 out of 39 pairs used).

Experimental protocols

Series I: Net acid-base fluxes

The objective of this series was to use exposure to external hypercapnia to identify differences in acid-base regulation resulting from social stress. Control ($N = 9$), dominant ($N = 6$) and subordinate ($N = 6$) fish (mass 208 ± 8 g, $N = 21$) were placed in individual opaque acrylic boxes (volume ~4 L) supplied with flowing, aerated water and allowed to recover for 24 h. The experimental period commenced with a 3 h normocapnic flux period that was followed by 24 h exposure to external hypercapnia (1% CO_2) incorporating three 3 h flux periods. A water sample (10 mL) was withdrawn at the beginning and end of each 3 h flux period. The initial water sample was taken after the water flow to the experimental chamber was shut off and the water adjusted to a set level. During flux periods, water in the experimental chambers was aerated directly with either air (normocapnia; -3 to 0 h) or 1% CO_2 in air (hypercapnia; 1 to 4 h, 5 to 8 h or 21 to 24 h). Following the initial normocapnic flux, experimental boxes were supplied with water from an equilibration column (nominal water PCO_2 = 7.5 Torr; see below) for 1 h prior to the first hypercapnic flux; between hypercapnic fluxes, experimental chambers were flushed with flowing hypercapnic water for

at least 1 h to eliminate waste accumulation. Net acid-base flux ($J_{net}H^+$) was calculated from measurements of net titratable alkalinity flux ($J_{net}TA$) and ammonia flux ($J_{net}NH_3$) in these water samples. Water samples were analyzed within 6 h of sampling.

To achieve hypercapnia (in all experimental series), a water equilibration column was gassed with a mixture of CO_2 and air (GF-3/MP Gas Mixing Flowmeter; Cameron Instruments, Port Aransas, TX, USA). The PCO_2 of water leaving the equilibration column was monitored using a CO_2 electrode (E201; Analytical Sensors, Sugarland, TX, USA) housed in a temperature-controlled cuvette and linked to a meter (PHM72 MK2 Digital Acid-Base Analyzer; Radiometer, Copenhagen, Denmark) and data acquisition system (Biopac with AcqKnowledge v3.7.3 software; Harvard Apparatus Canada, Saint-Laurent, QC, Canada). Water and/or gas flows to the equilibration column were adjusted to achieve a nominal final water PCO_2 of ~ 7.5 Torr.

Fish were terminally sampled at the end of the experimental period by exposure to a lethal dose of anaesthetic (ethyl-*p*-aminobenzoate; 0.5 mg mL^{-1}). Blood samples (~ 1.0 mL) were withdrawn and centrifuged at 3,200 *g* for 3 min to separate plasma and red blood cells. The plasma was flash frozen in liquid N_2 and stored at -80°C for later analysis of cortisol. Samples of gill and posterior kidney tissues were placed in ice-cold SEI buffer (250 mM sucrose, 10 mM Na_2EDTA, 50 mM imidazole, at a pH of 7.3), flash frozen in liquid N_2 and stored at -80°C for later analysis of Na^+,K^+-ATPase (NKA) and H^+-ATPase activities. To control for the effects of social status on ATPase activities, an additional group of control, dominant and subordinate fish were sampled as described above following the 24 h recovery period (i.e. without having been exposed to hypercapnia).

Series II: Chloride fluxes

The objective of this experimental series was to determine whether any differences due to social status occurred in net or unidirectional chloride fluxes under hypercapnia. Control (N = 6), dominant (N = 6) and subordinate (N = 6) rainbow trout (mass 238 ± 8 g, N = 18) were allowed to recover overnight in experimental chambers (volume ~2 L). Water flow to the experimental chambers was then stopped, the water level was adjusted to a set value, and the water was spiked with $^{36}Cl^-$ (1 μCi L^{-1}; specific activity 10.7 mg Cl mL^{-1}; American Radiolabeled Chemicals Inc.). Water samples (8 mL) were withdrawn after a 15 min mixing period and at the end of the 4 h flux period. During this normocapnic period, experimental chambers were vigorously aerated. The experimental chambers were flushed, and then two successive 4 h flux periods were carried out using a similar approach but under hypercapnic conditions, achieved by vigorously aerating the boxes with a mixture of 1% CO_2 in air. ^{36}Cl radioactivity in water samples was measured using liquid scintillation β-counting (LS6500 Multi-Purpose Scintillation Counter; Beckman Coulter Inc., Fullerton, CA, USA) to determine the unidirectional Cl^- influx rate, and water chloride concentrations were assessed colorimetrically to assess the net Cl^- flux rate (see Analytical Techniques). The difference between net flux and influx was taken to be the unidirectional Cl^- efflux rate.

Series III: Blood acid-base status

The goal of this experimental series was to determine the blood gas and acid-base status of arterial blood samples under hypercapnic conditions and in relation to social status. Control (N = 7), dominant (N = 7) and subordinate (N = 7) fish (mass 260 ± 8 g, N = 21) were cannulated and placed into individual experimental chambers (volume ~4 L). After a 24 h post-surgery recovery period, fish were subjected to a 3 h control and 24 h hypercapnic

exposure as in series I, except that rather than carrying out flux periods, blood was sampled. Blood samples (0.6 ml) were withdrawn into an air-tight heparinized syringe from the dorsal aortic cannula at -1.5 (normocapnia), 2.5, 6.5 and 22.5 h of hypercapnia. An equal volume of saline was returned to the fish after each sample withdrawal. Haematocrit, haemoglobin concentration, total O_2 content, and PO_2 were measured on whole blood; the remainder of the sample was then centrifuged and true plasma was used for the measurement of total CO_2 content and pH. Blood or plasma samples were held in air-tight syringes on ice between measurements.

Series IV: Cortisol response to hypercapnia

The goal of this experimental series was to document changes in circulating cortisol concentrations over the course of hypercapnic exposure within socially stressed trout (dominant and subordinate) and control fish. The experimental protocol was virtually identical to that of series III, except that plasma obtained from blood samples (0.3 mL) was flash frozen in liquid N_2 and stored at -80°C for later analysis of plasma cortisol concentrations (using a commercial RIA kit, MP Biomedical).

Analytical techniques

In series I, water samples (10 mL) were analyzed for net titratable alkalinity ($J_{net}TA - J_{net}NH_3$) within 6 h of sampling. $J_{net}TA$ was assessed by titrating 5 mL of the initial and final water samples from each flux period to pH 4.00 using 0.02 mol L^{-1} HCl and noting the difference in titrant added between samples. Acid was delivered using a Gilmont microburette (GS-1200 GE 2.0 ml; Fisher, Pittsburgh, PA, USA and pH was recorded (pHC3005-8 electrode and PHM201 pH meter; Radiometer Analytical) throughout the

titration. Samples were bubbled with N_2 gas for 15 min prior to titration and continuously throughout titration to ensure mixing and removal of CO_2 liberated by titration of HCO_3^- (McDonald and Wood, 1981). Ammonia excretion must also be considered as protons can be lost in the form of NH_4^+ from the gills or trapped as NH_4^+ in the water by NH_3 excreted from gills (McDonald and Wood, 1981). Consequently, ammonia levels of the initial and final water samples were analyzed using a micro-modification of the salicylate-hypochlorite colorimetric assay of (Verdouw et al., 1978). Therefore, net acid flux ($J_{net}H^+$) was calculated as the sum of titratable alkalinity ($J_{net}TA$) and ammonia flux ($J_{net}NH_3$) with signs considered.

A kinetic microplate assay was used to determine NKA (McCormick, 1993) and H^+-ATPase (NEM-sensitive ATPase activity; Lin and Randall, 1993) activities. Gill and kidney tissue samples were thawed on ice and homogenized using a hand-held power homogenizor (Pellet Pestle Motor; Kimble Kontes, Vineland, NJ, USA) following the addition of 250 µL of SEID (250 mM sucrose, 10 mM Na_2EDTA, 50 mM imidazole, and 0.1% v/v deoxycholic acid at pH 7.3). Samples were centrifuged for 30 s at 5,000 *g* and the supernatant was assayed in duplicate in 3 separate buffers: first simply reaction buffer, second in reaction buffer to which ouabain (0.5 mM) had been added, and finally in reaction buffer to which NEM (1 mM) had been added. Protein content of the same supernatant samples diluted 20-fold was measured using the bichinchonic acid method (BioRad) as described in the manufacturer's manual. Ouabain-sensitive ATPase (Na^+,K^+-ATPase) activity and NEM-sensitive ATPase (H^+-ATPase) activity were expressed in units of µmoles ADP mg^{-1} protein h^{-1} following comparison of rates in the presence and absence of ouabain or NEM.

In series II, chloride uptake rates were measured by the addition of $^{36}Cl^-$ (1 µCi L^{-1}; American Radiolabeled Chemicals Inc.) to each experimental chamber. Water samples were assessed for ^{36}Cl radioactivity by mixing 4 mL of each water sample to 20 mL of Bio-Safe II

cocktail (Research Products Intl., Mount Prospect, IL, USA) and using a β-counter (LS6500 Multi-Purpose Scintillation Counter; Beckman Coulter Inc.). Water samples were also analyzed for total chloride concentration using a method modified from Zall et al. (1956). The concentration of chloride was determined colorimetrically using mercuric thiocyanate and ferric nitrate. Unidirectional Cl^- influx was calculated as the disappearance of isotope from the water, using specific activity to convert ^{36}Cl radioactivity to Cl^- concentration and taking water volume, flux period and fish mass into account. Net Cl^- flux was calculated from the change in Cl^- concentration in the experimental chamber over the flux period, taking water volume and fish mass into account; a positive net flux value indicated net Cl^- uptake by the fish. Unidirectional efflux rates were calculated as the difference between net flux and unidirectional influx.

In series III, blood samples were analyzed for P_{O2}, haematocrit, haemoglobin concentration and total O_2 content. Blood P_{O2} was measured by inserting into the blood sample a fibre-optic O_2 electrode (AL300; Ocean Optics Sensors, Dunedin, FL, USA) connected to a spectrophotometer and data acquisition system (OOI Sensors Oxygen Measurement; Ocean Optics). Haematocrit was measured in duplicate using 60 μL of blood in a heparinized microcapillary tube (Fisher, Pittsburgh, PA, USA) centrifuged at 6,000 *g* for 6 min. Haemoglobin concentration ([Hb]) was measured in duplicate by adding 10 μL of blood to 5 mL of Drabkin's solution (Sigma, Mississauga, ON, CA). Absorbance was measured at 540 nm on an aliquot of this solution using a microplate spectrophotometer (340PC Spectramax; Molecular Devices, Sunnyvale, CA, USA) and compared to values obtained for haemoglobin standards. Blood total O_2 content was measured in triplicate on 20 μL samples using a blood content analyzer (Oxycon; Cameron Instruments). Following these measurements, the remainder of the blood sample was centrifuged (3,200 *g* for 3 min)

to yield plasma that was assessed for pH and total CO_2 content. Arterial pH (pH_a) was assessed using a pH electrode and calomel reference (E301 glass pH electrode; Cameron Instruments) housed in a temperature-controlled (13°C) low-volume pH chamber (Cameron Instruments) and connected to a PHM71 Acid-Base Analyzer (Radiometer). Total CO_2 content ($[CO_2]_{tot}$) was measured in duplicate on 50 μL samples using a total CO_2 analyzer (965 Carbon Dioxide Analyser; Corning Limited, Halstead, Essex, UK). Arterial PCO_2 and plasma $[HCO_3^-]$ were calculated by rearrangement of the Hendersen-Hasselbalch equation using values of αCO_2 and pK' appropriate for rainbow trout plasma (Boutilier et al., 1985).

Statistical Analysis

All data are presented as mean values ± standard error of the mean (SEM). Statistical significance was assessed by one-way repeated measures analysis of variance (RM ANOVA), by two-way RM ANOVA, or using a one-sample Student's *t*-test, as appropriate. With ANOVA, *post hoc* multiple comparisons tests (Bonferroni) were used to identify the source of any significant differences detected. All analyses used a significance level of 5% and were carried out with commercial software (SigmaStat v3.0; SPSS Scientific Inc.).

Results

Behavioural observations

Rainbow trout (*O. mykiss*; $N = 78$, mass = 236 ± 5 g, length = 27.1 ± 0.1 cm) were paired for five days. Because individual social status is assigned on the basis of behaviour scores, the scores of dominant and subordinate trout (Fig. 3-1) differed significantly (Student's *t*-test, $P < 0.001$), suggesting that a clear hierarchy formed in most pairs. Pairs with clear hierarchies yielded dominant ($N = 39$, mass = 243 ± 7 g, length = 27.3 ± 0.2 cm) and subordinate ($N = 39$, mass = 228 ± 6 g, length = 26.9 ± 0.2 cm) groups. Only one pair was excluded from further analysis because a clear hierarchy did not form (less than 0.5 points between the behaviour scores of the paired fish).

Series I Acid-base regulation

Control, dominant and subordinate trout were exposed to hypercapnia and net acid excretion ($J_{net}H^+$) was measured (Fig. 3-2) as the sum of the net excretion of titratable acid equivalents ($J_{net}TA$) and net ammonia excretion ($J_{net}NH_3$), signs considered. Fish were exposed to a nominal water PCO_2 of 7.6 Torr; PCO_2 measured during the initial two hypercapnic flux periods was close to the desired value (7.5 ± 0.02 and 7.90 ± 0.2 Torr, $N =$ 4 measurements from separate trials for each) but tended to rise during the overnight period when changes in water flow often occurred, averaging a significantly higher 9.8 ± 1.2 Torr (N = 4) for the final hypercapnic flux period. This increase in PCO_2 during the overnight period was not ideal. However, members of a pair were held under similar conditions and comparison of these conditions to that of the control individuals did not find any statistically significant differences (two-way RM ANOVA for the final sample period at 22.5 h for dominants vs controls, p = 0.727, dominants vs subordinates, p = 1.000 and subordinates vs

controls, p = 0.952). As a reference for responses of control, dominant and subordinate trout to hypercapnia, a separate group of control trout ($N = 9$) was held under normocapnia conditions for an equivalent period of time. Under normocapnic conditions, $J_{net}H^+$ remained constant (one-way RM ANOVA, $P = 0.586$) over the 24 h experimental period at a mean rate of 111.0 ± 15.3 μmol kg^{-1} h^{-1} (dashed line in Fig. 3-2). Net acid flux values for all groups were positive under control conditions (indicating net acid excretion; Fig. 3-2), and did not differ significantly (one-sample Student's *t*-test, $P > 0.05$ for all comparisons) from the mean net acid excretion value for the control group held under normocapnic conditions. $J_{net}H^+$ increased significantly in response to hypercapnic exposure within both control and dominant groups, but not among subordinate trout (Fig. 3-2; one-way RM ANOVA, P = 0.035, 0.004, 0.391 for control, dominant, and subordinate fish, respectively). Interestingly, control and dominant trout differed in $J_{net}H^+$ at the final flux (18-21 h of hypercapnia), where $J_{net}H^+$ in control trout returned to control levels whereas in dominant trout, the elevated net acid excretion was sustained at the final flux period (Fig. 3-2 A,B).

Breaking net acid flux down into its individual components of net titratable alkalinity ($J_{net}TA$; Fig. 3-3 B) and net ammonia flux ($J_{net}NH_3$; Figure 3-3 C), again, values for a control group held under normocapnic conditions remained constant over time (one-way RM ANOVA, P = 0.728 for $J_{net}TA$ and 0.724 for $J_{net}NH_3$) and are indicated on the figures as dashed lines for comparison. In subordinate fish, neither $J_{net}TA$ nor $J_{net}NH_3$ was altered significantly (one-way RM ANOVA, $P = 0.644$ and 0.241, respectively) by hypercapnic exposure, accounting for the absence of an effect on net acid excretion. Control and dominant trout appeared to differ in the strategies used to increase net acid excretion when exposed to hypercapnia (Fig. 3-3). Whereas dominant trout responded to hypercapnia with net acid excretion mediated by significant increases in $J_{net}TA$ (one-way RM ANOVA, P =

0.013) but not $J_{net}NH_3$ (one-way RM ANOVA, $P = 0.407$), control trout exposed to hypercapnia increased $J_{net}NH_3$ (one-way RM ANOVA, $P = 0.008$) but not $J_{net}TA$ (one-way RM ANOVA, $P = 0.072$). Control (normocapnic) flux rates for control, dominant and subordinate trout were compared to those for the control group held under normocapnic conditions. No differences were detected between the two control groups, but $J_{net}TA$ in dominant fish was significantly lower than the control value (one-sample Student's *t*-test, $P < 0.05$), while $J_{net}NH_3$ in subordinate fish was significantly higher than the control value (one-sample Student's *t*-test, $P < 0.05$) even though only one subordinate trout in all 39 pairs consumed 1 food pellet.

Na^+,K^+-ATPase (NKA) activity was quantified in gill and kidney tissues sampled at the end of 24 h exposure to either normocapnia or hypercapnia (Fig. 3-4). Gill NKA activity was affected by both social status and exposure condition (Fig. 3-4, two-way ANOVA, $P =$ <0.001, 0.877 and <0.001 for the effects of social status, exposure condition and the interaction between these factors, respectively). After 24 h of hypercapnic conditions, gill NKA activity in control fish was significantly lower than the value for control fish held under normocapnic conditions, unchanged in dominant trout, and significantly higher than the normocapnic value in subordinate fish. Under normocapnic conditions, control fish exhibited the highest gill NKA activity, whereas following 24 h of hypercapnia, gill NKA activity in subordinate fish was significantly higher than that in dominant or control trout (Fig. 3-4). By contrast, NKA activity in the kidney was unaffected by exposure to hypercapnia, but subordinate trout exhibited significantly lower renal NKA activity than control or dominant fish (Fig. 3-4, two-way ANOVA, P =0.033, 0.873 and 0.191 for the effects of social status, exposure conditions and the interaction between these factors, respectively).

H^+-ATPase activity was also quantified in both gill and kidney tissues following 24 h exposure to either normocapnia or hypercapnia (Fig. 3-5). Gill H^+-ATPase activity varied with respect to both social status and exposure condition (Fig. 3-5 A; two-way ANOVA, P = <0.001, 0.001, and 0.026 for the effects of social status, exposure condition and the interaction of these two terms, respectively). Gill H^+-ATPase activity levels were affected by exposure to hypercapnia only in subordinate trout, where gill H^+-ATPase activity was significantly elevated under hypercapnia exposure. Under normocapnic conditions, gill H^+-ATPase activity was highest in subordinate fish, an effect that was greatly magnified under hypercapnic conditions. Renal H^+-ATPase activity was unaffected by hypercapnic exposure or social status (two-way ANOVA, P = 0.455, 0.636, and 0.261 for the effects of social status, exposure condition and the interaction of these two terms, respectively).

Series II Chloride fluxes under control and hypercapnic conditions

Chloride fluxes were measured across three 4 h flux periods beginning with a normocapnic period followed by two consecutive hypercapnic flux periods (water PCO_2 values of 7.6 ± 0.1 and 7.7 ± 0.1 Torr; N = 5 for each time). Exposure to hypercapnia had a significant effect on net chloride flux (two-way RM ANOVA; $P < 0.001$ for sampling time, 0.054 for social status and 0.799 for the interaction of these two terms), causing net chloride loss in all groups during the first hypercapnic flux (Fig. 3-6). Although the effect of social status was not significant (two-way RM ANOVA, P = 0.054), there was a trend for control trout to exhibit net chloride uptake under normocapnic conditions while dominant and subordinate trout exhibited net chloride loss. Moreover, dominant and subordinate trout exhibited a tendency towards greater net chloride loss than control trout during the first hypercapnic flux period (Fig. 3-6).

Because in no case was there a significant effect of social status on movement of chloride ions, mean values across all three experimental groups are presented for net chloride flux (measured as the change in water chloride ion concentration over the course of the flux period), unidirectional chloride influx (measured isotopically), and unidirectional chloride efflux (the difference between net flux and influx; Fig. 3-7). Analysis of these data indicated that the significant net loss of chloride ions during the first hypercapnic flux (2-way RM ANOVA, P <0.001) reflected a significant reduction in chloride uptake (2-way RM ANOVA, P = 0.846, 0.049 and 0.949 for the effects of social status, sampling time and the interaction of these two terms, respectively) in the absence of any change in chloride efflux (2-way RM ANOVA, P =0.486, 0.699, 0.761 for the effects of social status, sampling time and the interaction of these two terms, respectively).

Series III Blood acid-base status during hypercapnia

To examine the effect of hypercapnic exposure on blood acid-base status, blood samples were collected from control, dominant and subordinate trout under normocapnic conditions and at time points in the hypercapnic exposure corresponding to the midpoint of flux periods used to measure $J_{net}H^+$. Water PCO_2 levels during hypercapnic exposure rose consistently (two-way RM ANOVA, P = 0.174, <0.001 and 0.003 for the effects of social status, sampling time and the interaction of these two factors, respectively) over the course of the 24 h exposure, averaging 7.9 ± 0.1, 10.2 ± 0.3 and 13.5 ± 0.6 Torr (N = 21 in each case) for the 2.5 h, 6.5 h and 22.5h measurement time points. In addition, dominant and subordinate fish were exposed to a significantly higher (14.6 ± 0.7 Torr, N = 14) final PCO_2 than were control fish (11.3 ± 0.9 Torr, N = 7). Although this difference was not desirable, making comparisons between dominant/subordinate and control trout at the final sampling

time somewhat difficult to interpret, comparisons between dominant and subordinate trout were not affected. In addition, to control for the effects of blood sampling alone, a separate group of control fish was subjected to an equivalent sampling protocol during 24 h of normocapnic exposure.

Rainbow trout sampled under normocapnic conditions for a 24 h experimental period showed the expected effects due to repeated blood sampling (Fig. 3-8) including significant declines in haematocrit (one-way ANOVA, $P < 0.001$), blood haemoglobin concentration (one-way ANOVA, $P < 0.001$) and blood oxygen content (one-way ANOVA, $P < 0.001$). Both haematocrit and haemoglobin increased transiently at the second sample time, but fell with additional blood withdrawal. Mean corpuscular haemoglobin concentration (MCHC), calculated as the ratio of haemoglobin concentration to haematocrit, increased over the experimental period (one-way ANOVA, $P < 0.001$). However, acid-base status was unaffected by repeated sampling under normocapnic conditions, with PCO_2 (1.58 ± 0.12), pH (7.89 ± 0.03) and plasma [HCO_3^-] (5.28 ± 0.09) remaining constant over time (one-way ANOVA, $P = 0.458$, 0.656, 0.996, respectively).

Patterns of change in indices of blood O_2 transport in control, dominant and subordinate trout during hypercapnia were very similar to those in the control fish held under normocapnic conditions, suggesting that these changes were the result primarily of repeated sampling (Fig. 3-9). These significant effects of repeated blood sampling were, however, in some cases, social status-specific (Fig. 3-9). Haematocrit responses were unaffected by social status (two-way RM ANOVA, $P < 0.001$ for the effect of time, $P = 0.43$ for the effect of social status, and $P = 0.359$ for the interaction of these two terms). Blood haemoglobin levels exhibited essentially the same pattern of response in fish exposed to hypercapnia as in the normocapnic control group (two-way RM ANOVA, $P = 0.034$ for the effect of social

status, $P < 0.001$ for the effect of time, and $P = 0.150$ for the interaction of these two terms). However, blood haemoglobin concentration was significantly higher in dominant than in control fish. Mean corpuscular haemoglobin concentration (MCHC) also exhibited these trends, but in this case subordinate and control fish differed significantly (two-way RM ANOVA, $P = 0.011$ for the effect of social status, $P < 0.001$ for the effect of time, and $P = 0.495$ for the interaction of these two terms). Blood oxygen content was also found to decline across the sampling period in all groups (two-way RM ANOVA, $P = 0.06$ for the effect of time, $P = 0.277$ for the effect of social status, and $P = 0.003$ for the interaction of these two terms). Interestingly, whereas blood O_2 content fell sharply in control trout at the first hypercapnic sampling point before recovering somewhat, blood O_2 content in both dominant and subordinate trout increased slightly at the first hypercapnic sample before falling with repeated sampling. This effect was significant in subordinate but not dominant fish.

Comparing indices of blood O_2 transport under resting conditions (Figure 3-8), results in a significant difference between subordinate and control normocapnic trout in haematocrit levels with subordinate trout having much lower hct (one-way ANOVA, P = 0.019). Blood haemoglobin concentration was significantly higher in control normocapnic group than in either control hypercapnic or subordinate groups (one-way ANOVA, P = 0002), however there were no differences in blood O_2 content or mean corpuscular haemoglobin concentration (Figure 3-8).

Blood acid-base status was determined by measurement of plasma PCO_2, pH, and bicarbonate ion concentration ($[HCO_3^-]$; Fig. 3-10). In general, blood acid-base status was influenced by exposure to hypercapnia, but not by social status. Plasma PCO_2 increased significantly in all groups upon exposure to hypercapnia (two-way RM ANOVA, $P = 0.854$

for the effect of social status, $P < 0.001$ for the effect of time, and $P = 0.015$ for the interaction of these two terms). Although statistical analysis revealed social status-specific differences in the elevation of blood PCO_2, the differences were subtle, consisting of a more rapid rise in PCO_2 in control fish than in either dominant or subordinate trout, and probably reflected the water PCO_2 profile rather than a phenomenon of physiological significance. Plasma PCO_2 levels determined from measurements of plasma pH and total CO_2 using the Henderson-Hasselbalch equation were substantially lower under hypercapnic conditions (~5.6 Torr at the final sample time) than expected on the basis of the level of hypercapnia to which the fish were exposed (nominally PCO_2 = 7.6 Torr). To establish the source of this difference, blood PCO_2 measurements were carried out on a subgroup of control, dominant and subordinate fish using a PCO_2 electrode connected to a blood gas analyzer. Measurements made in this fashion revealed actual PCO_2 values to be ~12.2 Torr (at the final sample time). As with the calculated values, measured PCO_2 values were unaffected by social status (2-way RM ANOVA, P =0.348, <0.001 and 0.3214 for the effects of social status, sampling time and the interaction of these two factors, respectively). Because this measurement approach required a significant volume of blood, making it impractical to combine with other blood measurements, these measurements were discontinued. The calculated PCO_2 values were clearly underestimating the actual PCO_2 values, probably owing to CO_2 loss from blood samples driven by the high partial pressure gradient between hypercapnic blood samples and air. However, all blood samples were handled in the same fashion, allowing comparisons among treatment groups (control, dominant and subordinate fish), and over time, to be made. Neither pH nor [HCO_3^-] levels were affected by social status (two-way RM ANOVA, for the effects of social status, time and the interaction of these two terms, respectively, P = 0.995, <0.001, and 0.555 for pH and P = 0.248, <0.001,

and 0.392 for [HCO_3^-]) and therefore mean data for all treatment groups were combined for presentation purposes (Fig. 3-10). Mean pH values exhibited a significant drop in response to hypercapnic exposure but had recovered to the normocapnic value by 24 h of hypercapnia. Data for the 6.5 h sample time for pH were rendered unusable by a calibration error with the pH meter. Reflecting the steady increase in water PCO_2, plasma [HCO_3^-] increased steadily and significantly over the course of the hypercapnic exposure, approximately tripling by 24 h. Data for blood acid-base status are summarized in a pH-bicarbonate diagram (Fig. 3-11). All groups responded to hypercapnia with an initial respiratory acidosis, blood pH falling along the buffer line, but by 24 h metabolic compensation had occurred in the form of an increase in [HCO_3^-] and hence pH at a constant PCO_2.

Series IV Cortisol responses during hypercapnic exposure

As expected, subordinate fish exhibited significantly (one-tailed 2-way RM ANOVA, P = 0.026, 0.809 and 0.537 for the effects of social status, sampling time and the interaction of these terms, respectively) elevated plasma cortisol concentrations in comparison to dominant or control fish (Fig. 3-12). Circulating cortisol concentrations were unaffected by exposure to hypercapnia.

Figure 3-1. Behaviour scores for dominant (N=39, mass = 243 ± 7 g, length = 27.3 ± 0.2 cm) and subordinate (N=39, mass = 228 ± 6 g, length = 26.9 ± 0.2 cm) rainbow trout (*Oncorhynchus mykiss*) used for subsequent experiments. Behaviour scores were assigned using principle components analysis of data gathered across a 5 day observation period; behaviours such as aggression, position and feeding were documented. A more positive score indicates more dominant behaviours such as high levels of aggression, while negative scores indicate more submissive behaviours such as poor position in the environment. Data are means ± s.e.m. Data were analyzed using a Student's t-test, and an asterisk indicates a significant difference from the dominant value ($P < 0.001$).

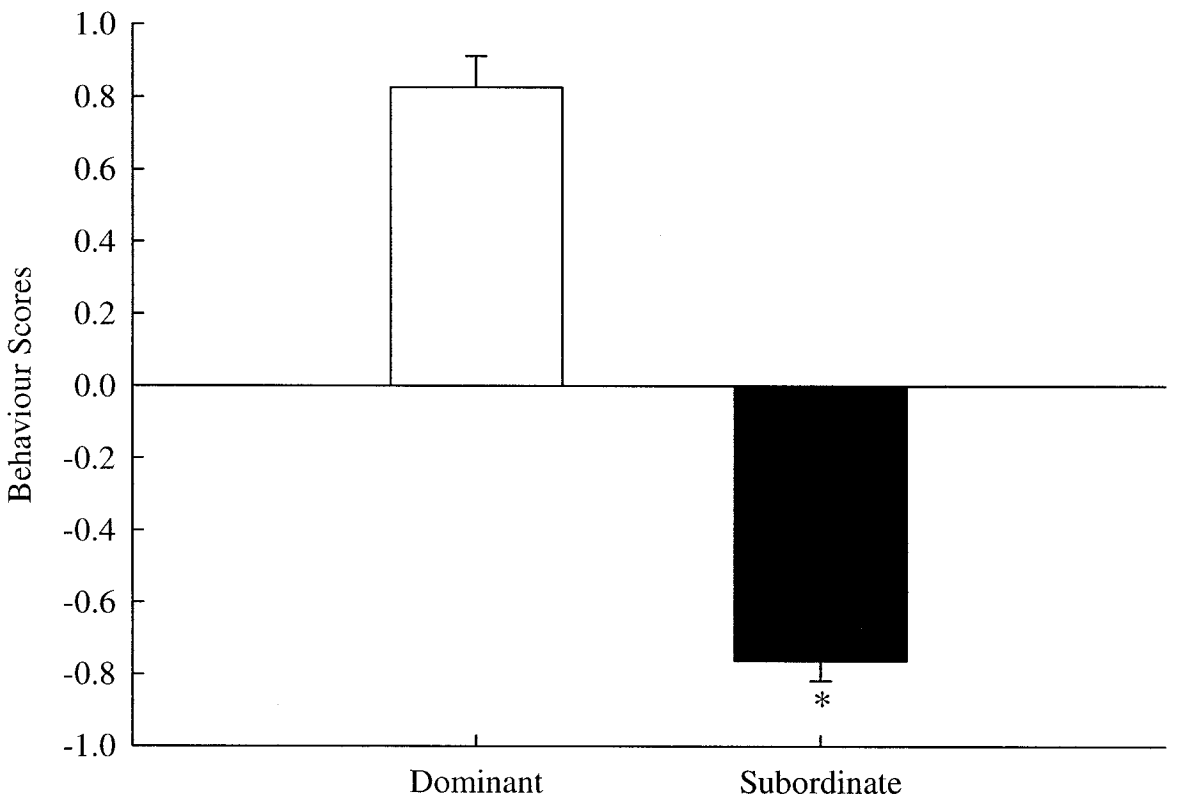

1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
Behaviour Scores
*
Dominant
Subordinate

Figure 3-2. The effect of exposure to hypercapnia (nominal water PCO_2 of 7.6 Torr) on net excretion of acidic equivalents ($J_{net}H^+$) in A) control ($N = 9$), B) dominant ($N = 6$), and C) subordinate ($N = 6$) rainbow trout (*Oncorhynchus mykiss*). $J_{net}H^+$ was calculated as the sum of $J_{net}TA$ and $J_{net}NH_3$ with signs considered. The dashed line in each panel indicates the mean value for a control group of trout that was exposed to normocapnia for 24 h ($N = 9$). Data are means ± 1 s.e.m. Diagonal hatching indicates data collected under control (normocapnic) conditions. Data for each treatment group were analyzed by one-way repeated measures ANOVA with sampling time as the factor of interest. Times that do not share a letter are significantly different from one another ($P = 0.035$, 0.004 and 0.397 for control, dominant and subordinate treatment groups, respectively).

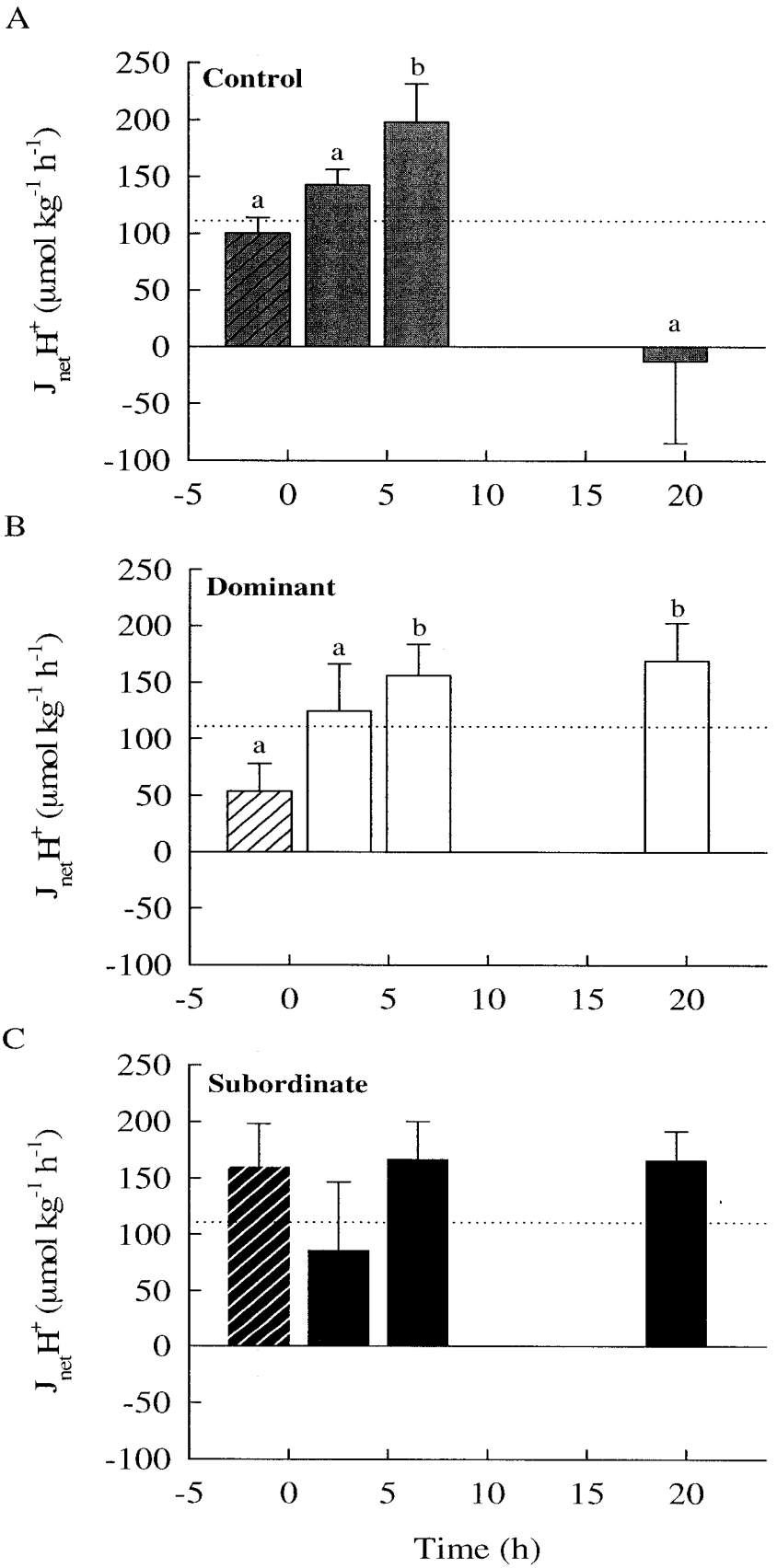

A
Control
B
Dominant
C
Subordinate
$J_{net}H^+$ (µmol kg^{-1} h^{-1})
250
200
150
100
50
0
-50
-100
-5
0
5
10
15
20
a
a
b
a
a
b
b
a
b
Time (h)

Figure 3-3. The effect of exposure to hypercapnia (nominal water PCO_2 of 7.6 Torr) on A) net excretion of acidic equivalents ($J_{net}H^+$), B) titratable net acid flux ($J_{net}TA$) and, C) net ammonia excretion ($J_{net}NH_3$) in control, dominant and subordinate rainbow trout (*Oncorhynchus mykiss*). $J_{net}H^+$ was calculated as the sum of $J_{net}TA$ and $J_{net}NH_3$ with signs considered; data for $J_{net}H^+$ are repeated from Fig. 3-2 for comparison. The dashed lines indicate the mean values for each variable for a control group of trout exposed to normocapnic conditions for 24 h ($N = 9$). Data are means ± 1 s.e.m.; $N = 9$ for controls and $N = 6$ for both dominant and subordinate trout. Data for the normocapnic flux period prior to the onset of hypercapnia are indicated by diagonal hatching. Data for each treatment group were analyzed by one-way repeated measures ANOVA with sampling time as the factor of interest. Time points within a group that share a letter are not significantly different from one another; different letters were used for different treatment groups for clarity (i.e. control a, b and dominant x, y). For panel A, see Fig. 3-2. For panel B, $P = 0.072$, 0.013 and 0.644 for control, dominant and subordinate treatment groups, respectively. For panel C, $P =$ 0.008, 0.407 and 0.241 for control, dominant and subordinate treatment groups, respectively. A one-sample Student's *t*-test was used to compare values for the normocapnic flux period to the mean value for the control fish held under normocapnic conditions; an asterisk indicates a treatment group that differed significantly ($P < 0.05$) from the mean value.

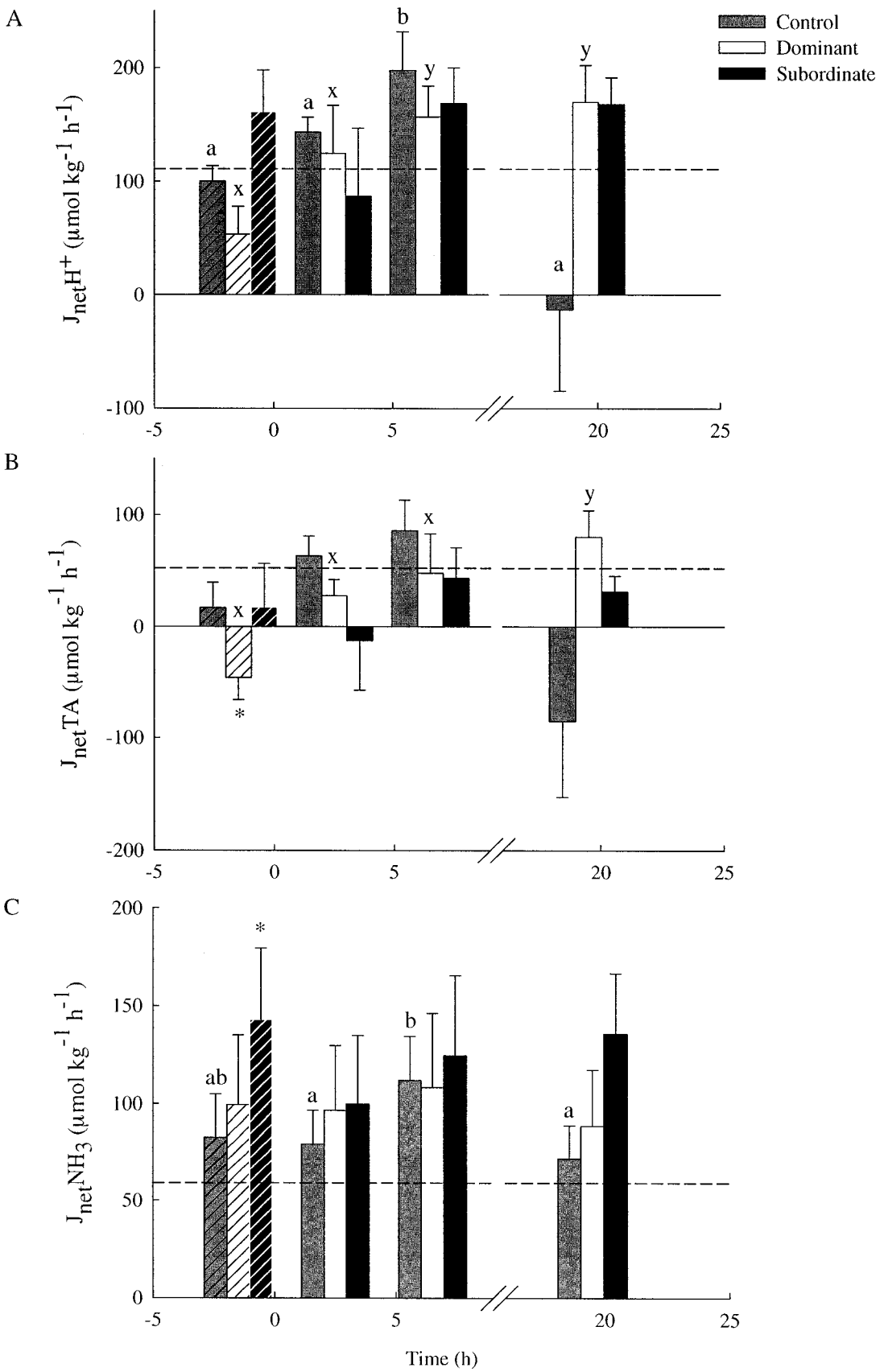

A
B
C
Control
Dominant
Subordinate
$J_{net}H^+$ (µmol kg^{-1} h^{-1})
$J_{net}TA$ (µmol kg^{-1} h^{-1})
$J_{net}NH_3$ (µmol kg^{-1} h^{-1})
Time (h)
200
100
0
-100
-200
150
50
-5
5
20
25
a
x
b
y
ab
*

Figure 3-4. The effect of 24 h of normocapnia or hypercapnia (nominal water PCO_2 of 7.6 Torr) on Na^+,K^+-ATPase activity in A) gill and B) kidney of control, dominant, and subordinate rainbow trout (*Oncorhynchus mykiss*). Data are means ± 1 s.e.m.; $N = 8$ for controls in both conditions $N = 7$ and 8 for dominants under control and hypercapnic conditions, respectively: and $N = 6$ and 7 for subordinates under control and hypercapnic conditions, respectively. Diagonal hatching indicates data collected under normocapnic conditions. Data were analyzed by two-way ANOVA with exposure condition (normocapnic or hypercapnic) and social status (control, dominant, or subordinate) as factors. In panel A, significant differences within a social status are indicated by asterisks, while differences among social categories within an exposure condition group are indicated by the use of different letters (a, b, c for normocapnia, x, y for hypercapnia). In panel B, significant effects of social status are indicated by the use of different letters (A, B, C). *P* values for the effect of social status, exposure condition and the interaction of these two factors, respectively, were A <0.001, 0.877, and <0.001, and B 0.033, 0.873, and 0.191.

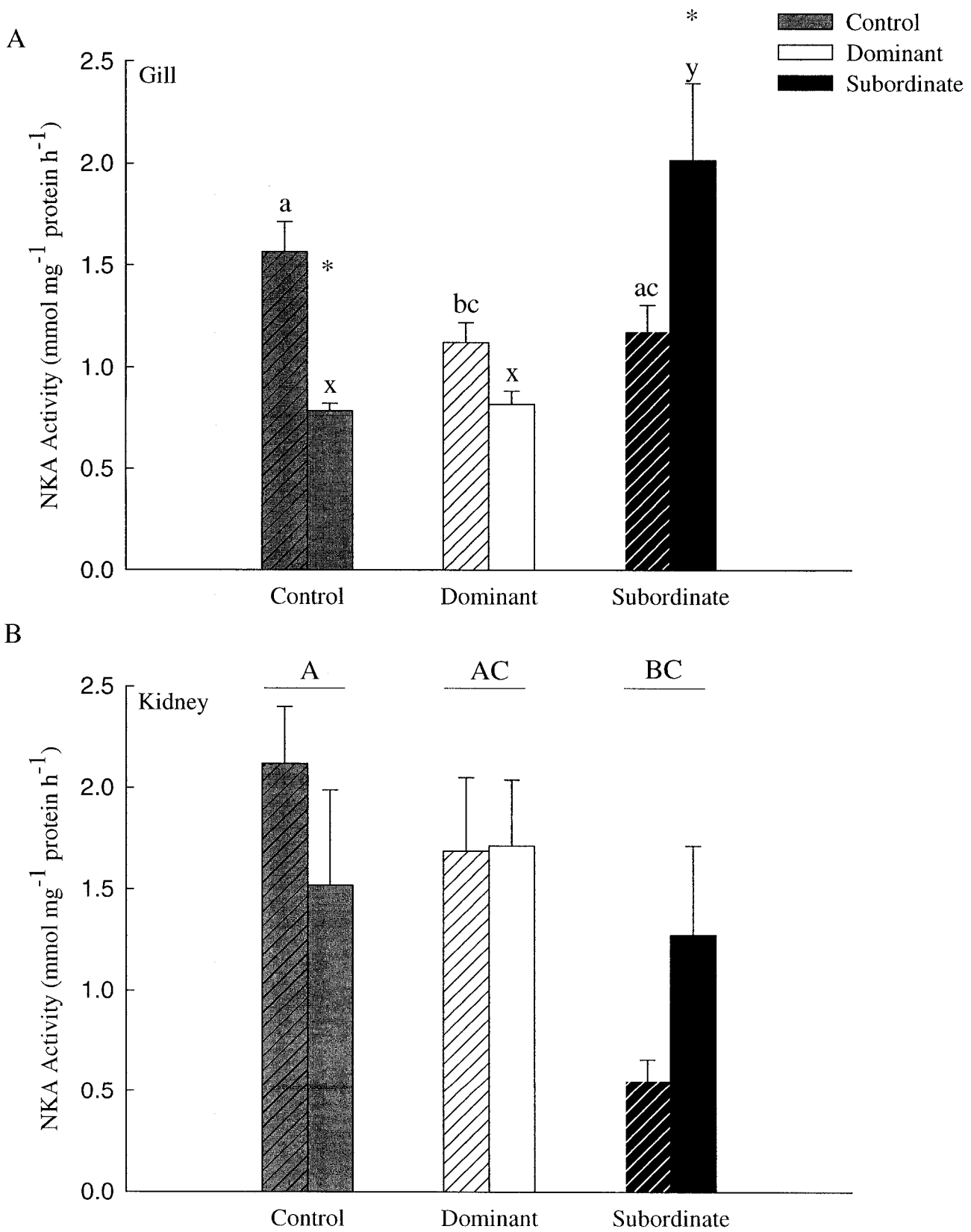

A
Gill
Control
Dominant
Subordinate
NKA Activity (mmol mg-1 protein h-1)
2.5
2.0
1.5
1.0
0.5
0.0
a
*
x
bc
x
ac
*
y
Control
Dominant
Subordinate
B
Kidney
A
AC
BC
NKA Activity (mmol mg-1 protein h-1)
2.5
2.0
1.5
1.0
0.5
0.0
Control
Dominant
Subordinate

Figure 3-5. The effect of 24 h of normocapnia or hypercapnia (nominal water PCO_2 of 7.6 Torr) on H^+-ATPase activity in A) gill and B) kidney of control, dominant, and subordinate groups of rainbow trout (*Oncorhynchus mykiss*). Data are means ± 1 s.e.m.; $N = 8$ for controls under both conditions, $N = 7$ and 8 for dominants under control and hypercapnic conditions, respectively, and $N = 7$ for subordinates under both conditions. Diagonal hatching indicates data collected under normocapnic conditions. Data were analyzed by two-way ANOVA with exposure condition (normocapnia or hypercapnia) and social status (control, dominant, or subordinate) as factors. In panel A, significant differences within a social status are indicated by asterisks, while differences among social categories within an exposure condition group are indicated by the use of different letters (a, b, c for normocapnia, x, y for hypercapnia). *P* values for the effect of social status, exposure condition and the interaction of these two factors, respectively, were A <0.001, 0.001, and 0.026 and, B 0.455, 0.636, and 0.261.

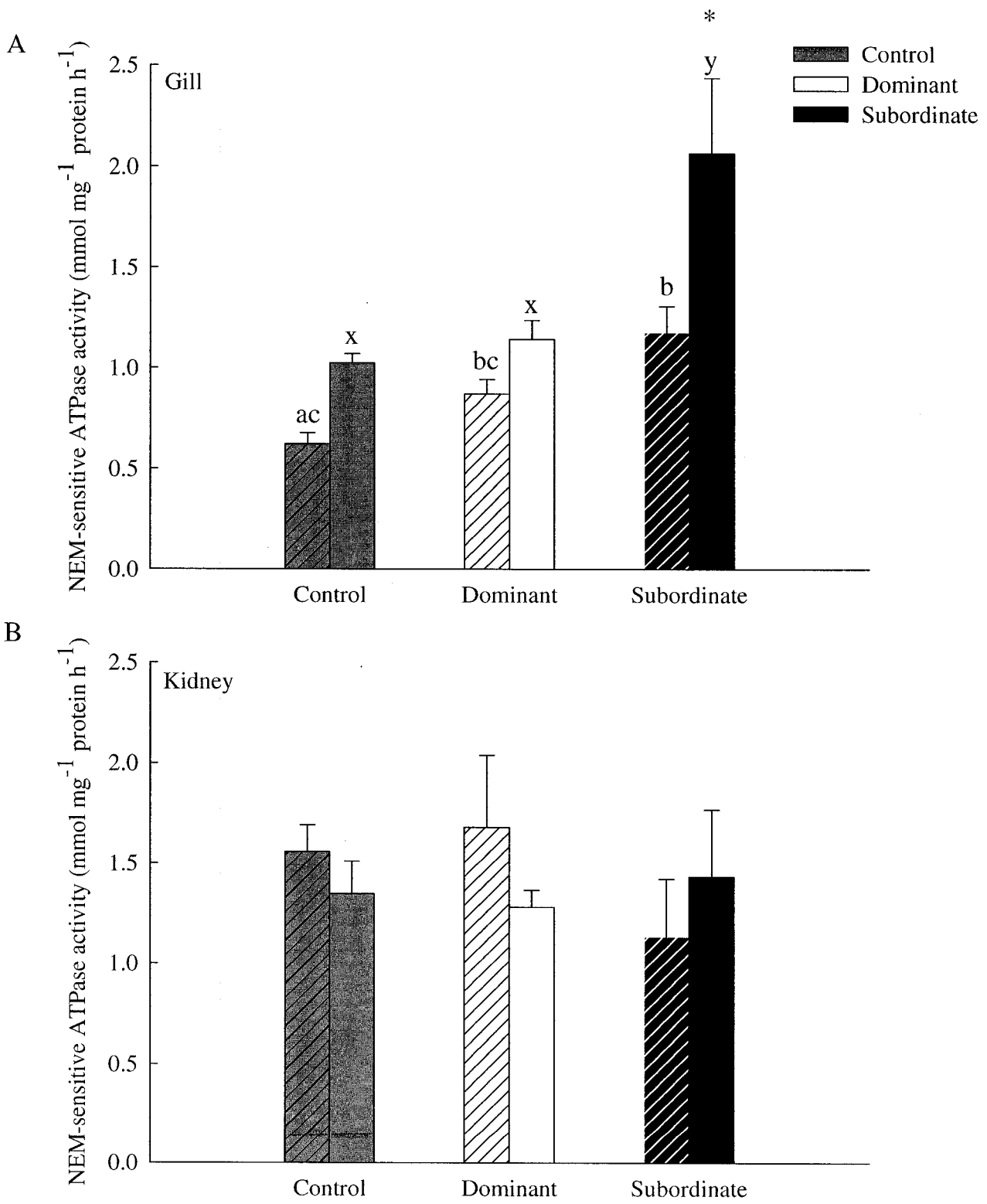

A
Gill
NEM-sensitive ATPase activity (mmol mg-1 protein h-1)
2.5
2.0
1.5
1.0
0.5
0.0
ac
x
bc
x
b
*
y
Control
Dominant
Subordinate
Control
Dominant
Subordinate
B
Kidney
NEM-sensitive ATPase activity (mmol mg-1 protein h-1)
2.5
2.0
1.5
1.0
0.5
0.0
Control
Dominant
Subordinate

Figure 3-6. The effect of hypercapnic exposure (nominal water PCO_2 of 7.6 Torr) on net chloride ion flux within control, dominant, and subordinate rainbow trout (*Oncorhynchus mykiss*). Data are means ± 1 s.e.m.; $N = 5$ for all groups. Diagonal hatching indicates data collected under normocapnic conditions. Data were analyzed by two-way repeated measures ANOVA with sampling time and social status (control, dominant, or subordinate) as factors. For sampling time, groups (in the absence of a significant effect of social status or a significant interaction term) that share a letter are not significantly different from one another. P values for the effect of social status, exposure condition and the interaction of these two factors, respectively, were 0.054, <0.001 and 0.799.

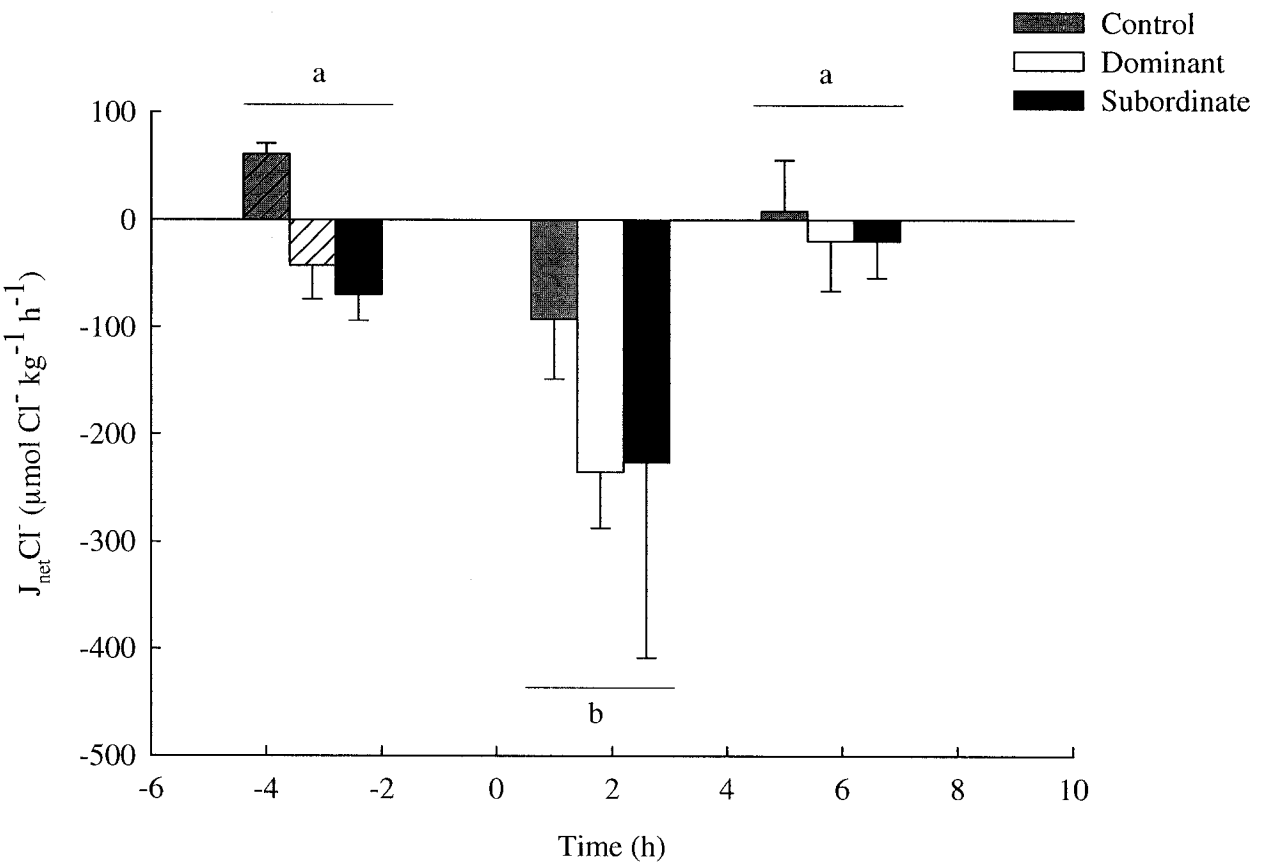

Control
Dominant
Subordinate
a
a
b
100
0
-100
-200
-300
-400
-500
-6
-4
-2
0
2
4
6
8
10
Time (h)
$J_{net}Cl^-$ (µmol Cl^- kg^{-1} h^{-1})

Figure 3-7. The effect of hypercapnic exposure (nominal water PCO_2 of 7.6 Torr) on A) unidirectional Cl^- influx, B) net chloride flux ($J_{net}Cl^-$), and C) unidirectional Cl^- efflux in rainbow trout (*Oncorhynchus mykiss*). Data are means for grouped data from control, dominant and subordinate fish ± 1 s.e.m.; $N = 15$. Diagonal hatching indicates data collected under normocapnic conditions. Data were analyzed using two-way repeated measures ANOVA with sampling time and social status (control, dominant, or subordinate) as factors. Groups that share a letter are not significantly different from one another. The data in panel B) are repeated from Fig. 3-6 but plotted as the mean value across all treatment groups rather than being broken down by treatment group; statistics for these data are reported in Fig. 3-6. *P* values for effect of social status, time and the interaction of these two factors, respectively, were 0.846, 0.049, and 0.949 for panel A and 0.486, 0.696, and 0.761 for panel C.

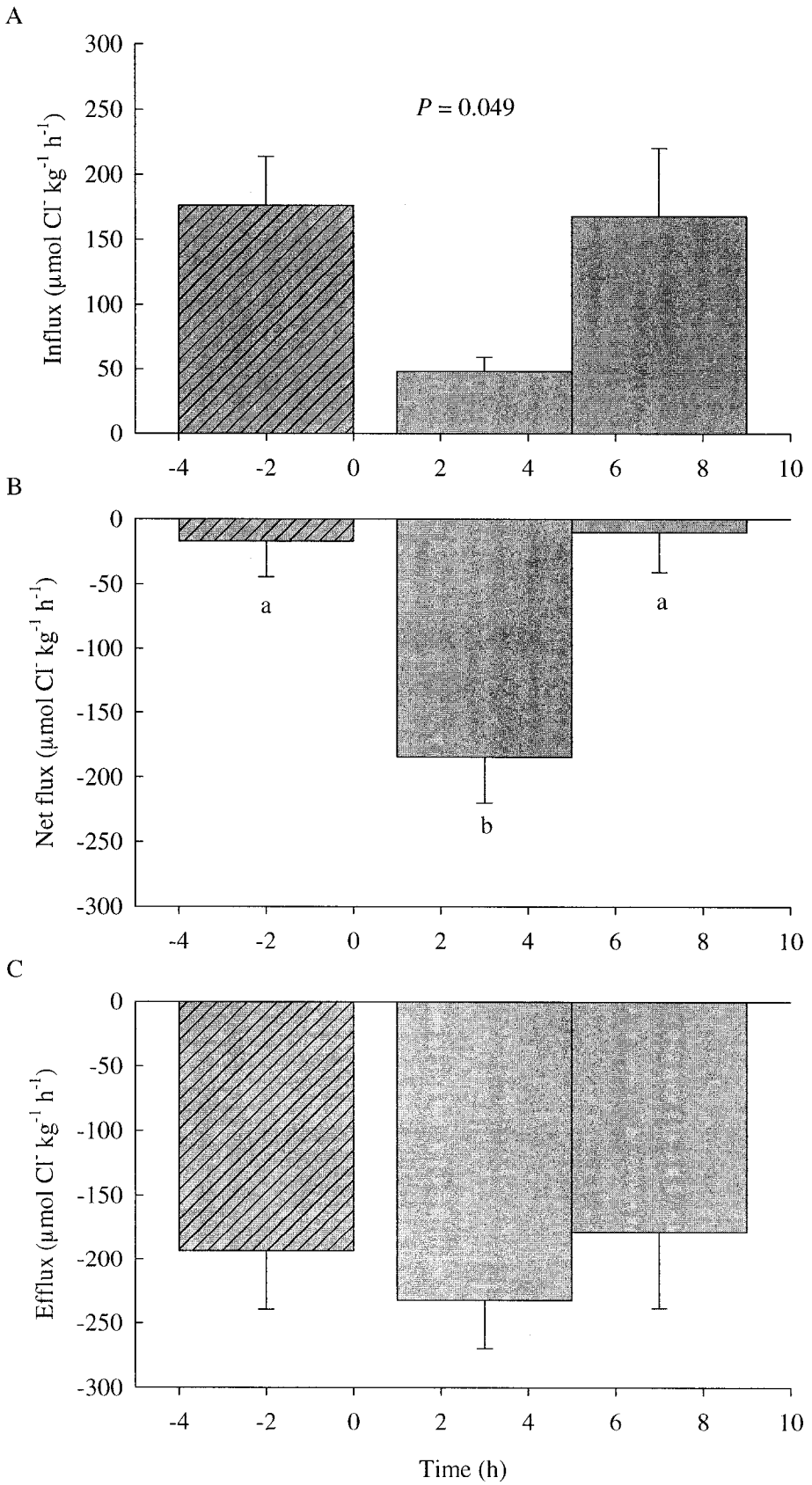

A
300
250
200
150
100
50
0
Influx (µmol Cl⁻ kg⁻¹ h⁻¹)
P = 0.049
-4
-2
0
2
4
6
8
10
B
0
-50
-100
-150
-200
-250
-300
Net flux (µmol Cl⁻ kg⁻¹ h⁻¹)
a
b
a
C
Efflux (µmol Cl⁻ kg⁻¹ h⁻¹)
Time (h)

Figure 3-8. Indices of blood O_2 status in arterial blood in rainbow trout (*Oncorhynchus mykiss*) exposed to normocapnia for 24 h. Parameters measured included A) haematocrit, B) oxygen content, C) haemoglobin concentration, and D) mean corpuscular haemoglobin content (MCHC). Data are means ± 1 s.e.m ($N = 6$) and were analyzed by one-way repeated measures ANOVA with time as factor. Significant ($P < 0.001$ for all variables) differences among sampling times are indicated by the use of different letters. Pre sample data (-1.5 h) for all groups (control normocapnic, control hypercapnic, dominant, and subordinate) were analyzed by one-way ANOVA. Values that were significantly different from subordinate group indicated by asterisks and values that were significantly different from control hypercapnic group indicated by a cross. *P* values between groups for haematocrit, oxygen content, haemoglobin concentration, and mean corpuscular haemoglobin content (MCHC), respectively, were 0.019, 0.157, 0.002, and 0.106.

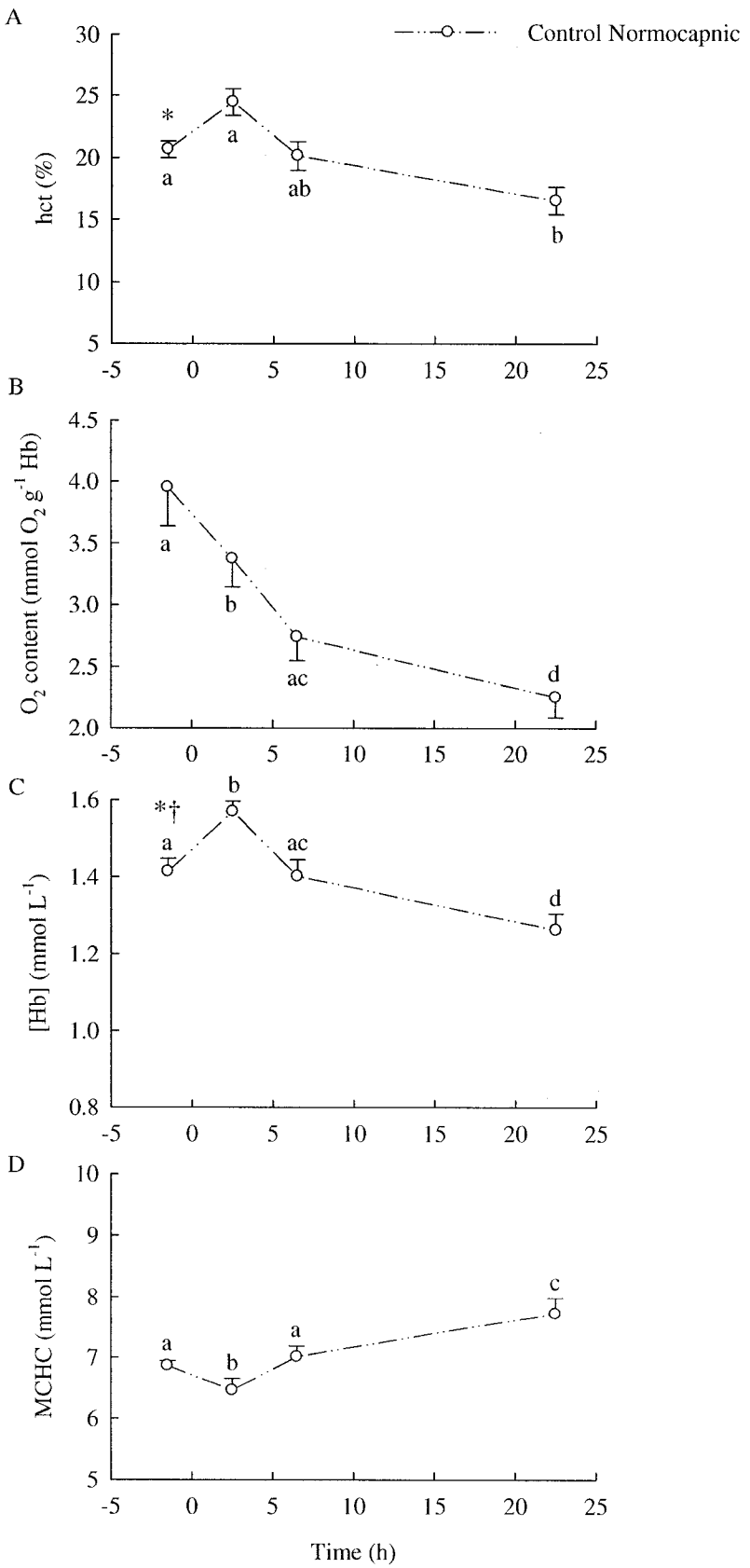

A
Control Normocapnic
hct (%)
B
O_2 content (mmol O_2 g^{-1} Hb)
C
[Hb] (mmol L^{-1})
D
MCHC (mmol L^{-1})
Time (h)

Figure 3-9. Indices of blood O_2 status in arterial blood in rainbow trout (*Oncorhynchus mykiss*) exposed to hypercapnia (nominal water PCO_2 of 7.6 Torr). Parameters including A) haematocrit, B) oxygen content, C) haemoglobin concentration, and D) mean corpuscular haemoglobin content (MCHC) were measured in control, dominant and subordinate trout. Data are means ± 1 s.e.m for each group (N = 6-7 for all groups) except in A), where mean values over all groups are presented ± 1 s.e.m; N = 21. The dotted line marks the start of the hypercapnic exposure. All data were analyzed by two-way repeated measures ANOVA with sampling time and social status (control, dominant, or subordinate) as factors. Groups that share a letter are not significantly different from one another. Small letters indicate significant differences within a treatment group as a function of sampling time. Large letters (A, B and C) indicate differences with sampling time for the combined data for control, dominant and subordinate trout, whereas large letters (X and Y) indicate differences due to social status for the combined data from all sampling times (in both of these cases, no significant interactions occurred between social status and sampling time). P values for the effect of social status, time and the interaction of these two factors, respectively, were 0.043, <0.001, and 0.359 for A, 0.277, 0.06, and 0.003 for B; 0.034, <0.001, and 0.15 for C, and 0.001, < 0.001, and 0.495 for D.

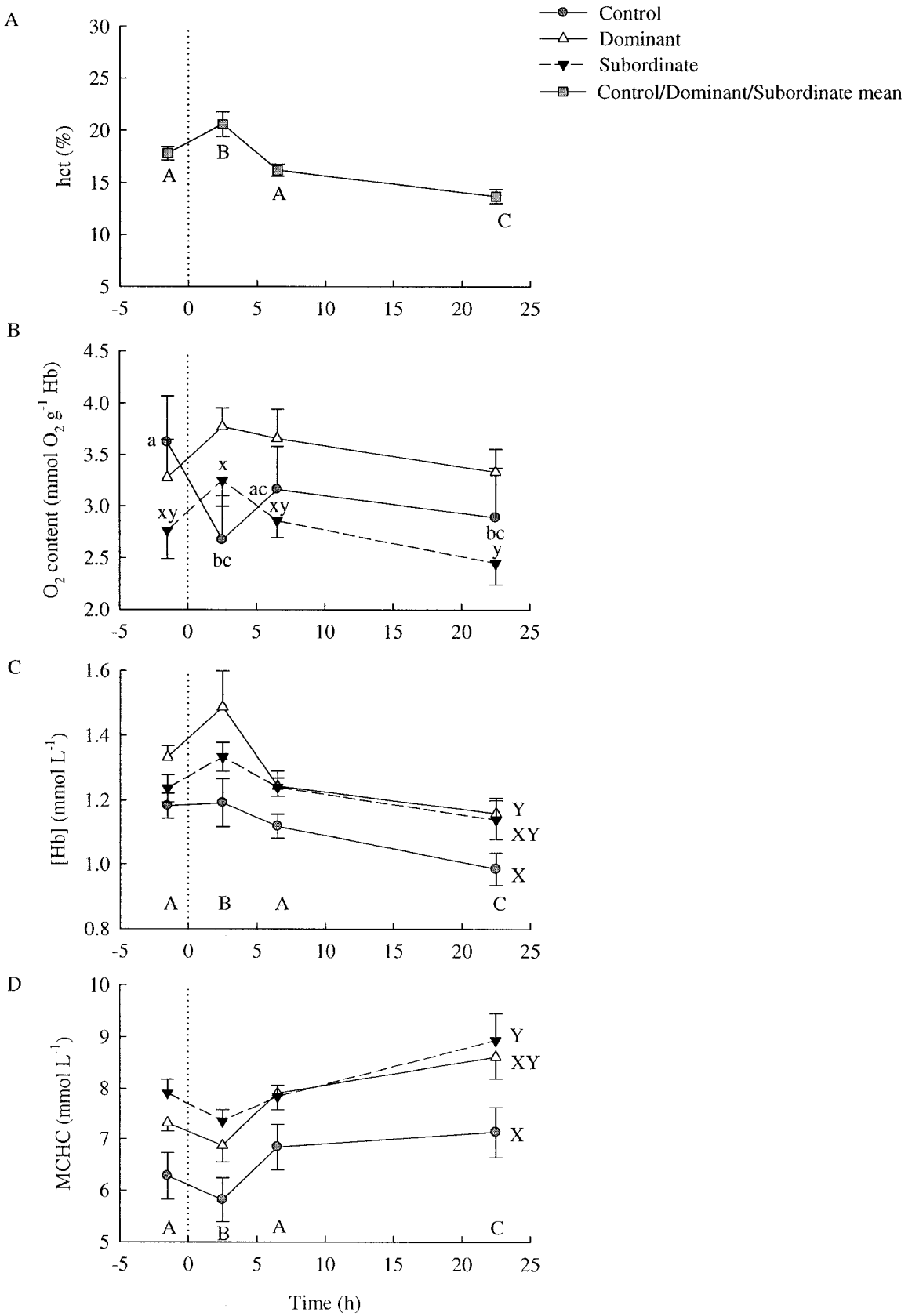

A
B
C
D
Control
Dominant
Subordinate
Control/Dominant/Subordinate mean
hct (%)
O_2 content (mmol O_2 g^{-1} Hb)
[Hb] (mmol L^{-1})
MCHC (mmol L^{-1})
Time (h)
a
x
ac
xy
bc
y
Y
XY
X
A
B
A
C

Figure 3-10. Blood acid-base status in rainbow trout (*Oncorhynchus mykiss*) exposed to hypercapnia (nominal water PCO_2 of 7.6 Torr). A) Plasma PCO_2, B) pH and C) $[HCO_3^-]$ were measured in control, dominant and subordinate trout. The dotted line marks the start of the hypercapnic exposure. The dashed lines indicate the mean values for each variable for a control group of trout exposed to normocapnic conditions for 24 h (N = 6). Values are means ± 1 s.e.m. (N = 6-7 for all groups). Data were analyzed by two-way repeated measures ANOVA with sampling time and social status (control, dominant, or subordinate) as factors. Groups that share a letter are not significantly different from one another. Small letter were used to indicate significant effects of sampling time within a treatment group, whereas large letters were used to indicate significant effects of sampling time across all treatment groups (i.e. where the interaction of the two factors was not significant). P values for the effect of social status, the effect of time and the interaction of these two factors, respectively, were 0.854, <0.001, and 0.015 in A, 0.995, <0.001, and 0.555 in B, and 0.248, <0.001, and 0.392 in C.

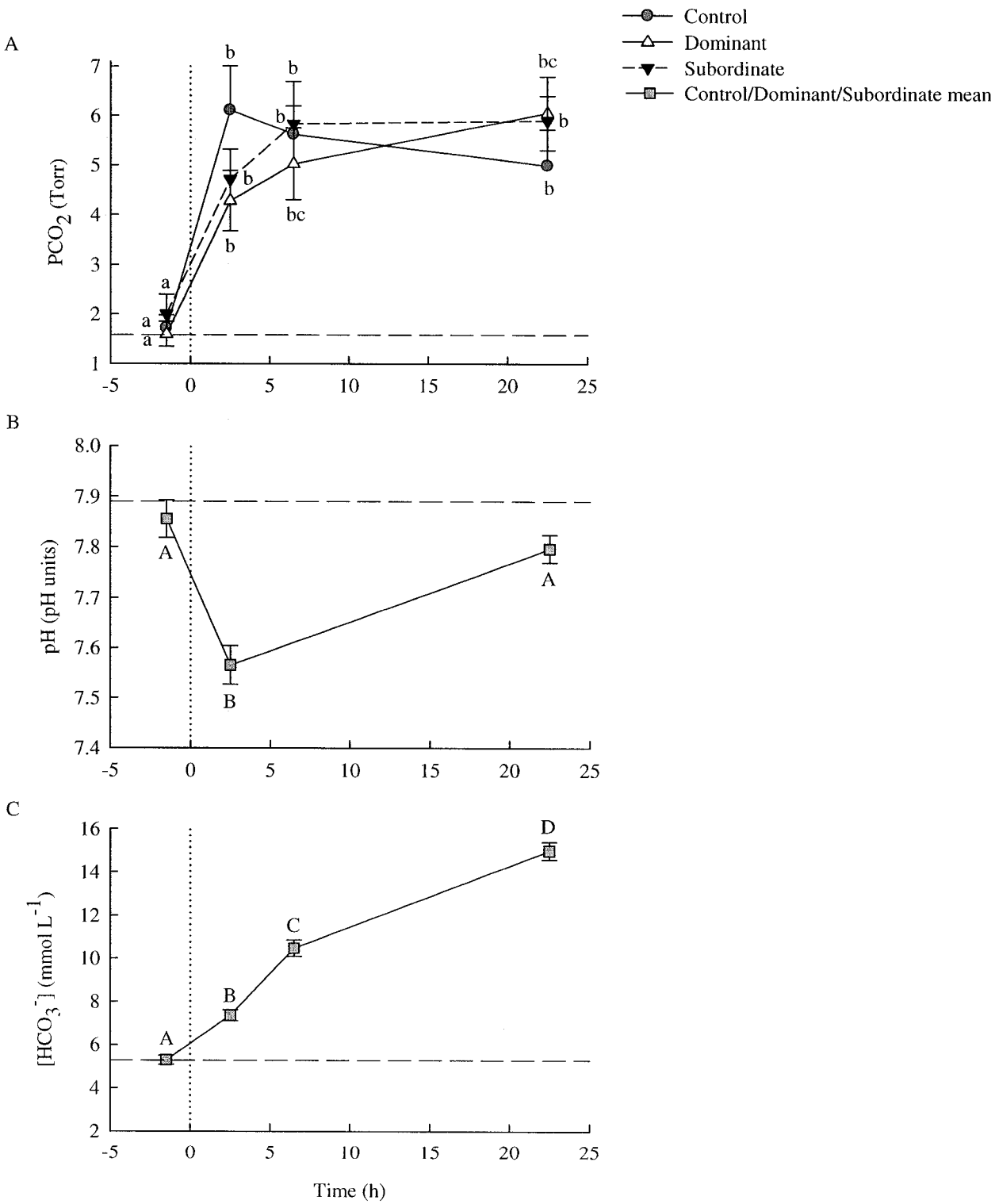

Control
Dominant
Subordinate
Control/Dominant/Subordinate mean
A
B
C
PCO_2 (Torr)
pH (pH units)
$[HCO_3^-]$ (mmol L^{-1})
Time (h)

Figure 3-11. A pH-bicarbonate diagram depicting responses of blood acid-base status to hypercapnia (nominal water PCO_2 of 7.6 Torr) in control, dominant, and subordinate rainbow trout (*Oncorhynchus mykiss*; $N = 7$ for all groups). The initial normocapnic value is indicated as A, B represents 2.5 h of hypercapnic exposure, and C represents 22.5 h of hypercapnia. Values in all cases are means ± 1 s.e.m. The PCO_2 for a given combination of pH and $[HCO_3^-]$ was calculated using the Henderson-Hasselbalch equation and the appropriate values for pK' and αCO_2 (Boutilier et al. 1984). Buffer lines for rainbow trout whole blood were constructed using buffer values derived by Wood et al. (1982). The solid line shows the passive buffer line for the blood (slope = 8.6 mmol L^{-1} pH $unit^{-1}$). Note that pH values are missing for the third sample period due to a pH meter calibration error. However, PCO_2 values for the third sample were estimated from expected pH values obtained by averaging pH values for the second and final samples, the expectation being that pH was recovering from its lowest value towards the final value at the time of the third sample period.

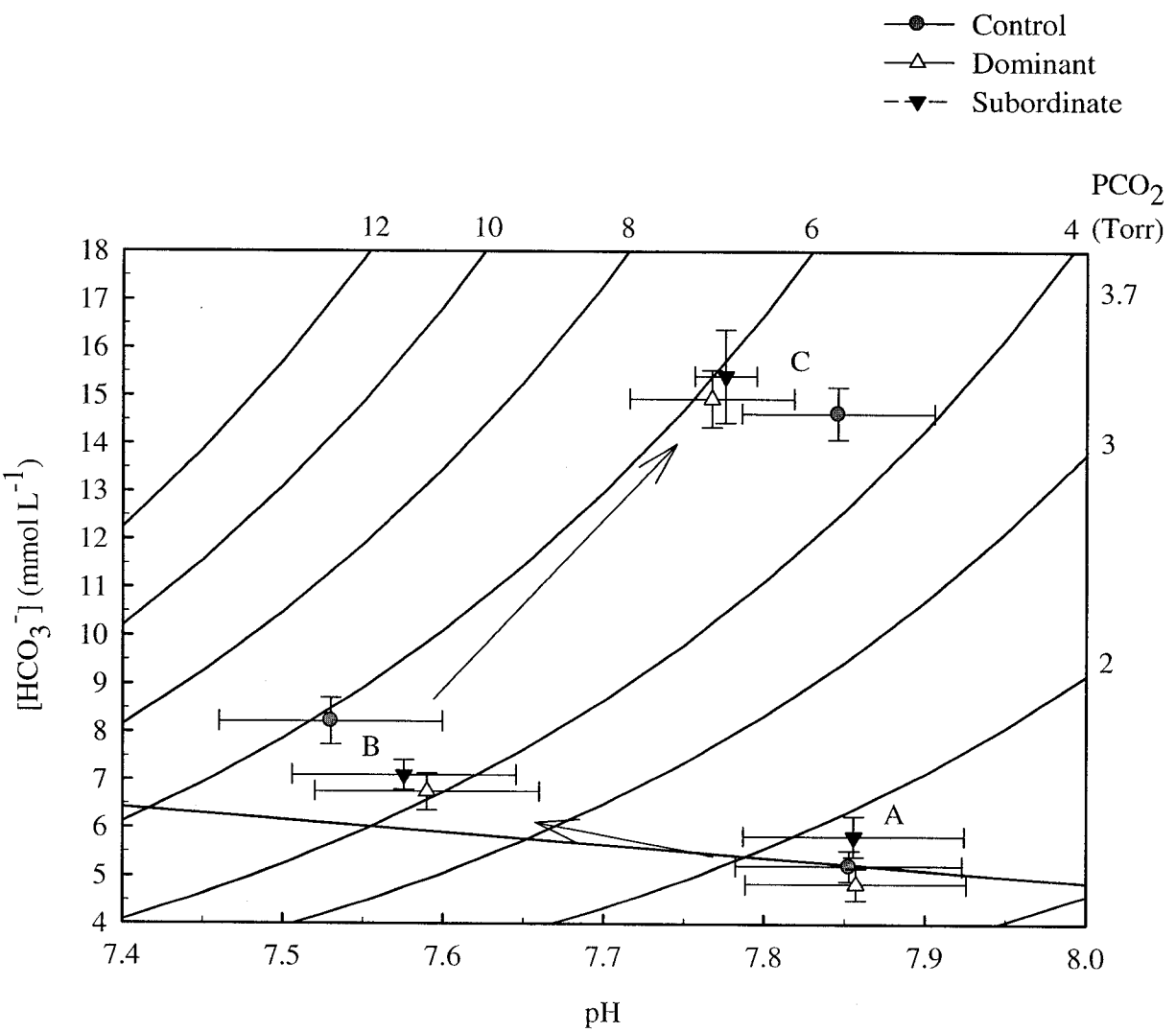
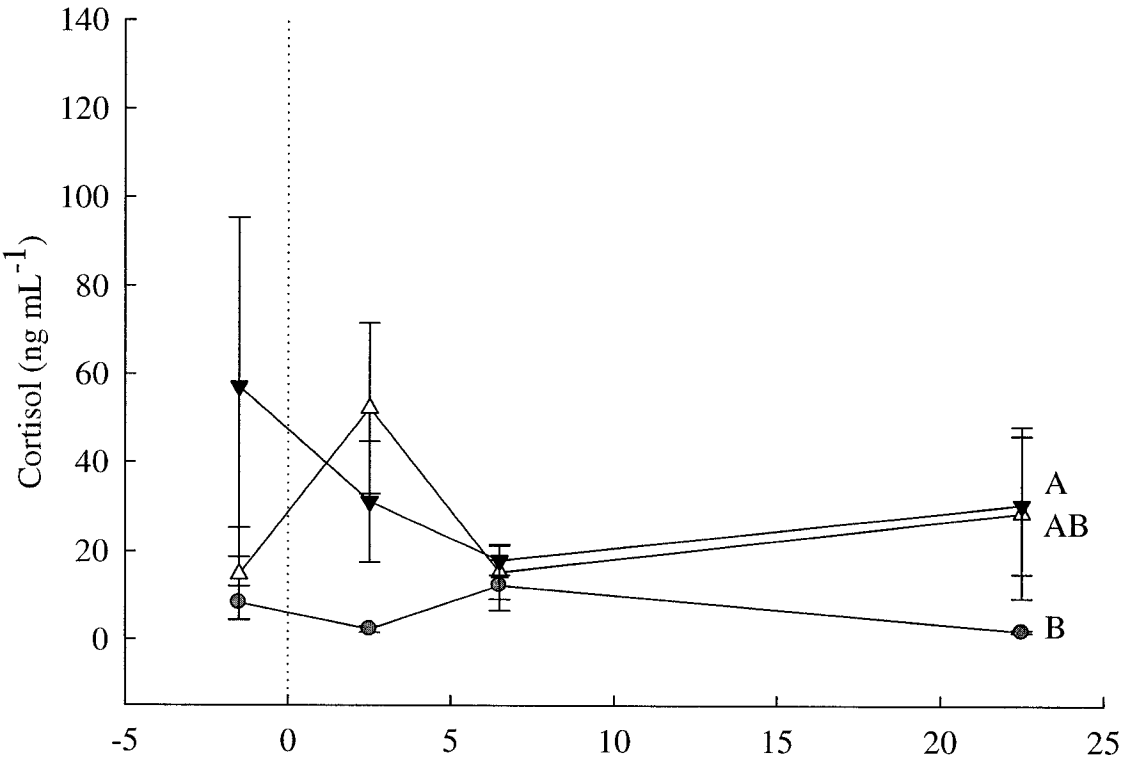

Control
Dominant
Subordinate
PCO2
(Torr)
12
10
8
6
4
3.7
3
2
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
[HCO3-] (mmol L-1)
7.4
7.5
7.6
7.7
7.8
7.9
8.0
pH
A
B
C

Figure 3-12. The effect of social status on circulating cortisol concentrations during exposure to hypercapnia (nominal water PCO_2 of 7.6 Torr) for control, dominant, and subordinate rainbow trout (*Oncorhynchus mykiss*). Data are means ± 1 s.e.m.; $N = 3$ for controls and subordinates, and $N = 6$ for dominants. The dotted line indicates the start of the hypercapnic exposure. Data were analyzed by two-way repeated measures ANOVA with social status (control, dominant, subordinate) and sampling time as the two factors (one-tailed two-way RM ANOVA, $P = 0.026$, 0.809 and 0.537 for the effects of social status, sampling time and the interaction of these terms, respectively. Groups that share a letter are not significantly different from one another.


140
120
100
80
60
40
20
0
Cortisol (ng mL-1)
-5
0
5
10
15
20
25
A
AB
B

Discussion

In contrast to the extensive literature on social status in terms of behavioural changes and physiological changes (Metcalfe et al., 1989; McCarthy et al., 1992; Adams et al., 1998, Overli et al., 1999a; Sloman et al., 2001), considerably less is known about the future impact of physiological changes. The objective of this study was to understand how social status impacts the ability of rainbow trout to cope with an acid-base challenge. Sloman et al. (2003b, 2004) found that Na^+ uptake was elevated in subordinate rainbow trout to compensate for the increased Na^+ loss (both branchial and renal) that fish of low social status exhibited. Consequently, net Na^+ balance in subordinate fish was not affected. However, the greater Na^+ fluxes in subordinate fish might be expected to have knock-on effects such as impacts on metabolic rate (i.e. elevated metabolic rate needed to support greater rates of ion transport), impacts on ion transport pathways (e.g. greater expression of transport proteins needed to support greater rates of ion transport), and impacts on processes dependent upon ion transport (e.g. acid-base balance). For example, Sloman et al. (2003b, 2004) found that increased Na^+ uptake in subordinate fish also resulted in significantly higher copper uptake and tissue burden over dominant counterparts, because copper enters the fish via Na^+ uptake pathways. Any effects on metabolic rate may be very difficult to assess as the energetic cost of osmoregulation in salmonids is thought to account for 1% of resting metabolism (Eddy, 1982; Morgan and Iwama, 1991). The present study aimed to investigate another physiological process that is dependent upon ion transport, that is, to understand how social status impacts the acid-base regulatory response of rainbow trout experiencing the acid-base challenge imposed by exposure to hypercapnia. The intimate integration of ionic and acid-base regulation in fish suggests that changes in one will have consequences for the other (reviewed by Evans et al., 2005).

The responses of rainbow trout to hypercapnia are well established (Cameron & Randall, 1972; Janssen & Randall, 1975). Upon introduction of hypercapnia, internal pH drops as internal CO_2 levels rise. To combat this respiratory acidosis, HCO_3^- ions are accumulated in the plasma to allow plasma pH to be corrected while PCO_2 remains high (Cameron & Randall, 1972; Cameron, 1978; Holeton et al., 1983; Claiborne & Heisler, 1984; Perry et al., 1987a; Wood, 1991; Choe & Evans, 2003; reviewed by Evans, 2005). In rainbow trout, the increase in plasma HCO_3^- is accomplished primarily by limiting branchial Cl^- uptake (which limits HCO_3^- loss) and to some extent by increasing branchial Na^+ uptake (which increases net acid excretion) (Perry et al., 1987a). Changes in branchial transfer of net acidic equivalents reflect a variety of processes, including remodeling of the gill epithelium (reviewed by Goss et al., 1995, 1998) and changes in the expression of ion transport and related proteins (reviewed by Perry & Fryer, 1997; Perry et al., 2003a; Perry & Gilmour, 2006). Renal responses to hypercapnia are also observed (Perry et al., 1987b). These responses focus on increasing HCO_3^- reabsorption from the filtrate, as in the absence of this response, branchial accumulation of HCO_3^- ions would be futile (Wood et al., 1999; Perry et al., 2003a; Georgalis et al., 2006b).

Because the compensatory response of rainbow trout to hypercapnia rests largely upon reductions in branchial Cl^- uptake/HCO_3^- loss and in previous work, effects of social status on Na^+ but not Cl^- movements were examined (Sloman et al., 2003b, 2004), an important goal of the present study was to examine the impact of social status upon Cl^- fluxes under both normocapnic conditions and in response to hypercapnia. Assuming that the effects of social rank on Cl^- movement would be similar to those on Na^+ movement (i.e. increased Cl^- uptake to compensate for greater Cl^- loss), then subordinate fish might be predicted to have greater difficulty than dominant fish in compensating for a respiratory

acidosis because of the need to lower Cl^- uptake. In addition, Sloman and colleagues (2004) reported higher urine flow rates in subordinate than dominant fish, that would, in turn, be expected to result in greater renal HCO_3^- loss, as was the case for renal Na^+ loss (Sloman et al., 2004). During hypercapnic exposure, greater renal HCO_3^- loss would be expected to become particularly significant, again attenuating the ability of subordinate fish to compensate for the respiratory acidosis. Unexpectedly, however, the results of the present study indicated that subordinate fish did not differ from dominant or control trout in unidirectional Cl^- uptake rates under control conditions, yet despite their comparable Cl^- flux rates, subordinate trout were unable to increase net acid excretion in response to hypercapnia. The absence of an increase in net acid excretion during hypercapnia did not, however, prevent subordinate trout from accumulating HCO_3^-, as plasma HCO_3^- concentrations in subordinate trout were comparable to those of dominant or control fish at all sample times. In addition to the impact of low social status on net acid excretion during hypercapnia, subordinate fish exhibited differences in branchial, and to some extent, renal, NKA and proton pump activities under both normocapnic and hypercapnic conditions. Finally, the profile of cortisol responses was different in subordinate fish from that of dominant or control trout. Taken as a whole, these results suggest that subordinate fish are able to regulate body fluid pH during respiratory acidosis, but that this regulation may come at a greater cost than is the case in dominant or control fish. These key points are explored in greater detail below.

Unidirectional Cl^- uptake rates in subordinate fish in the present study did not differ from those in dominant or control trout. This result was somewhat surprising because Sloman et al. (2004) reported Na^+ uptake rates for subordinate fish that were approximately double those of dominant trout. Subordinate fish demonstrated greater Na^+ efflux (Sloman et

al., 2003b, 2004) that necessitated a compensatory increase in Na^+ influx to attain Na^+ balance. Sloman et al (2004) attributed the greater Na^+ loss of subordinate fish to stress-induced changes in gill permeability coupled with diminished renal Na^+ reabsorption, both effects that would be expected to impact Cl^- movements similarly. The observation that Cl^- uptake rates in subordinate fish did not differ from those in dominant or control trout suggests, by contrast, that the effects of social status on Na^+ movements observed by Sloman et al. (2004) were, in fact, specific to Na^+ as opposed to being a reflection of general permeability phenomena. That is, that social stress impacts Na^+/H^+ exchange without altering Cl^-/HCO_3^- exchange. In accordance with this hypothesis, subordinate trout in the present study exhibited branchial H^+-ATPase activity that was significantly elevated over that of control trout under normocapnic conditions, and significantly elevated over that of either control or dominant trout under hypercapnic conditions. Ion loss can result from a variety of changes at the branchial level including remodeling of the gill epithelium to decrease surface area resulting in greater permeability of ions, along with an increase in ventilation rate that results in increased ion loss (reviewed by Goss et al., 1995, 1998). Why low social status is associated with increased Na^+ efflux that in turn drives Na^+ influx remains to be determined. Moreover, if this result holds up to further scrutiny (i.e. simultaneous measurement of unidirectional Na^+ and Cl^- fluxes in dominant vs subordinate fish), it could provide an excellent model for work focused on elucidating the molecular mechanisms of ion transport in fish gills and the cellular localization of such transport proteins (see below).

Despite Cl^- fluxes being comparable to those of dominant and control trout, subordinate fish appeared to be unable to elevate net acid excretion during hypercapnia. However, net acid excretion in subordinate fish under normocapnic conditions was the

highest of any group examined, although not significantly different from the value for the normocapnic control fish. The relatively high net acid excretion of subordinate fish was driven by a normocapnic net ammonia excretion value that was significantly higher than that of the normocapnic control group. This elevated ammonia excretion was surprising given that subordinate trout rarely consumed food during the 5 day observation period (only one subordinate trout in all 39 pairs consumed 1 food pellet). The elevated ammonia excretion of subordinate trout remains to be investigated, but is probably related to the metabolic effects of subordinate social status. Metabolic rate is elevated in fish of low social status (Sloman et al., 2000c) and at the same time, subordinate fish are dependent upon on-board energy reserves (DiBattista et al., 2006). These effects contribute to the negative specific growth rates commonly observed in subordinate salmonids (Sloman et al., 2000a, 2000b, Barton, 2002, reviewed in Gilmour et al., 2005a). Under these conditions, use of muscle protein to meet energy demands may account for the elevated ammonia excretion of subordinate trout. In keeping with this hypothesis, subordinate trout exhibited significantly higher activities than dominant or control trout of particular aminotransferase enzymes (e.g. alanine aminotransferase) in both red and white muscle after 5 days of pairing (DiBattista et al., 2006).

Thus, net acid excretion under normocapnic conditions may be relatively high in subordinate trout due to either or both of elevated ammonia excretion (this study) and elevated Na^+ influx with consequent H^+ loss (Sloman et al. 2004). A possible consequence of relatively high net acid excretion under normocapnic conditions may be the capacity to compensate for respiratory acidosis without further elevation of net acid excretion, although both the marked elevation of branchial H^+-ATPase activity and the significant reduction in Cl^- uptake in subordinate trout under hypercapnic conditions argue against this conclusion.

An alternative possibility is that branchial net acid excretion was elevated in subordinate fish during hypercapnic exposure (accounting for the elevation of branchial H^+-ATPase activity and the reduction in Cl^- uptake) but that this increase was masked by a simultaneous increase in renal base loss. Sloman et al. (2004) documented increased urine flow rates and reduced renal Na^+ reabsorption in subordinate trout, phenomena that could result in greater renal base loss in subordinate trout, particularly under hypercapnic conditions where the filtered base load is greatly increased owing to elevated plasma HCO_3^-. Moreover, subordinate trout in the present study exhibited significantly lower renal NKA activity than control trout, an effect that could account in part for the reduced renal Na^+ reabsorption observed by Sloman et al. (2004). Reabsorption of filtered HCO_3^- ions depends on proton excretion into the filtrate, a process that is in part Na^+-dependent owing to the involvement of Na^+/H^+ exchange mechanisms (Ivanis et al., 2008b). Separation of net acid-base fluxes into branchial and renal components would help to address the question of whether renal base loss is enhanced by subordinate social status, particularly under hypercapnic conditions. However, such experiments require that the fish be fitted with a urine catheter (e.g. see Curtis and Wood, 1991) and in deciding upon an experimental protocol, the stress associated with this procedure was deemed to be of concern (see below).

Although subordinate fish appeared to be unable to elevate net acid excretion during hypercapnia, these fish regulated arterial blood pH as effectively as dominant or control trout, through the accumulation of HCO_3^-. Assessment of arterial blood status during exposure to hypercapnia required cannulation of the fish, and it is possible that the stress associated with surgery masked any subtle effects of social status per se. Several points argue against this possibility. First, cannulation of the dorsal aorta in trout is a minimally invasive and relatively rapid procedure (e.g. Axelsson & Fritsche, 1994). Indeed, in a prior

study, dominant and subordinate trout re-paired following cannulation of the dorsal aorta and subsequent recovery, were found to have behaviour scores that were not significantly different from those measured prior to surgery (Thomas, 2005). Second, indices of blood oxygenation status in cannulated subordinate trout were found to differ from those of dominant and/or control fish in both the present study (see below) and in previous work (Thomas, 2005). Thus, it appears that blood acid-base status in rainbow trout is not affected by social rank, and it is unclear why the failure of subordinate fish to elevate net acid excretion under hypercapnic conditions did not translate into effects on blood acid-base status.

Blood oxygenation status did, however, demonstrate effects of both social stress and sampling. An increase in haematocrit and [Hb] in response to hypercapnia in all groups likely reflected red blood cell (RBC) mobilization from the spleen in response to the impact of acidosis on blood oxygen transport in a fish species that exhibits both Bohr and Root effects (Eddy et al., 1977; Thomas & LeRuz, 1982; Perry et al., 1987a). The subsequent drop in haematocrit in all groups reflected repeated sampling, an effect that is well documented (Soivio et al., 1975). Interestingly, dominant trout exhibited higher [Hb] levels than control fish, a situation that may have stemmed from stress-induced RBC release from the spleen during hierarchy formation. Both dominant and subordinate fish mobilize catecholamines during hierarchy formation (Thomas and Gilmour, 2006). Subordinate trout also exhibited a trend towards elevated [Hb], although values for this group did not differ significantly from those for control trout. In previous work (Peters and Schwarzer, 1985), it was reported that subordinate social status was associated with decreased production of new RBCs and increased RBC destruction, and these factors may have limited the ability of subordinate trout to benefit from stress-induced RBC mobilization from the spleen.

The stress of subordinate social status resulted in elevated circulating cortisol levels in subordinate trout (Fig. 3-12), a response that has been well documented in other studies (Pottinger & Pickering, 1992; Sloman et al., 2001, 2002; reviewed in Gilmour et al., 2005). The cortisol levels of subordinate trout in the present study were somewhat lower than those observed in some other studies (e.g. Thomas & Gilmour, 2006). The level of aggression within a pairing can impact levels of circulating cortisol (Winberg & Lepage, 1998; Sloman et al., 2000a), and while the pairs in this study had divergent behaviour scores indicating strong hierarchy formation, they did not exhibit high levels of aggression (personal observations). Cortisol levels in control and dominant trout averaged 15-17 ng mL^{-1}, values slightly higher than those expected for unstressed trout (0-5 ng ml-1; Pickering and Pottinger, 1989), and probably reflecting the stress associated with cannulation and/or confinement in an experimental chamber. In response to the additional, acute stress of exposure to hypercapnia, subordinate trout did not further increase circulating cortisol levels as was observed by Ivanis et al. (2008b) for rainbow trout (that had not undergone social interactions) exposed to a similar level of hypercapnia. It is possible that the ability of subordinate trout to mount a cortisol response to an additional, acute stressor was compromised by the effects of chronic cortisol elevation, an effect that has been documented previously in subordinate salmonids (Overli et al., 1999; Sloman et al., 2002) as well as in salmonids exposed to other chronic stresses (Vijayan and Leatherland, 1990; Balm and Pottinger, 1995; Brodeur et al., 1997, 1998; Hontela, 1998, Benguira and Hontela, 2000; Laflamme et al., 2000; Wilson et al., 1998) or to experimentally elevated exogenous cortisol (Balm & Pottinger, 1995; Barton et al., 1987). Additionally or alternatively, the cortisol profile of subordinate fish exposed to hypercapnia may have reflected the competing effects of a tendency for cortisol to rise in response to hypercapnia, but to fall in response to the

lengthening separation from the dominant fish. Plasma cortisol concentrations in subordinate fish might be expected to fall following separation from the dominant fish, but the time course of such a response does not appear to have been reported in the literature.

Future work and perspectives

The main conclusion of the present study is that subordinate fish appear to compensate for the respiratory acidosis incurred by exposure to hypercapnia as well as dominant or control trout do, based primarily upon the similarity of plasma pH regulation under hypercapnia across all three experimental groups. However, the results of the present study taken in conjunction with those of Sloman et al (2003b, 2004) on the effects of social status on Na^+ regulation suggest that there are subtle effects of social status on ionic and acid-base regulation in rainbow trout that may be worthy of further study. Two avenues that warrant investigation are to develop more comprehensive frameworks that describe the molecular mechanisms that underlie ion/acid-base equivalent transport, and characterize the responses of dominant versus subordinate trout to a metabolic acidosis.

Considerable controversy remains concerning the molecular mechanisms responsible for Na^+ and Cl^- uptake, and hence, respectively, H^+ and HCO_3^- excretion, at the freshwater fish gill, as well as the localization of these mechanisms into gill epithelial cell types. Na^+ may enter the animal through either an apically-located epithelial Na^+ channel (ENaC; but note that there is no evidence to date for the existence of an ENaC gene in the fish genomes that have been elucidated to date) coupled to an apical H^+-ATPase, or via an electroneutral exchange of Na^+ and H^+ mediated by an apical Na^+/H^+ exchanger (NHE), with Na^+ exiting the gill epithelium at the basolateral surface through Na^+,K^+-ATPase (reviewed by Hwang and Lee, 2007). Recent work increasingly favours a significant role for Na^+/H^+ exchange

mechanisms (Avella & Bornancin, 1989; Lin & Randall, 1991; reviewed in Marshall, 2002). For example, NHE3 expression was localized to the apical membrane of gill MR cells in a unique freshwater teleost, the Osorezan dace (*Tribolodon hakonensis*; Hirata et al., 2003) that lives in water of pH 3.4-3.8. This study found increased expression of NHE3 upon acclimation to highly acidic water, suggesting that it may play a role in acid secretion (Hirata et al., 2003). Moreover, Ivanis et al. (2008a) have localized NHE2 and NHE3 expression to a subset of MR cells in the gills of rainbow trout. Chloride ions are thought to be taken up in exchange for HCO_3^- via apical anion exchangers, and transferred across the basolateral membrane via a Cl^- channel down the electrical gradient, a mechanism that couples Cl^- uptake and base excretion (Perry & Randall, 1981; Chang & Hwang, 2004; reviewed by Evans et al., 2005). Recent work on rainbow trout (Goss et al., 2001), zebrafish (Lin et al., 2006) and tilapia (Hiroi et al., 2005), among others, suggests that a multiplicity of ion-transporting cell types exists in the fish gill, and identifying the complement of ion transporting proteins available in a given cell types remains challenging (reviewed by Hwang and Lee, 2007). The use of subordinate rainbow trout, which appear to exhibit elevated Na^+ but not Cl^- movement, might provide insight into this complex picture. Presumably, Na^+ transport pathways would be up-regulated in the gills of subordinate fish, while those for Cl^- would not.

The present study examined the responses of dominant and subordinate trout to respiratory acidosis, an acid-base challenge that, as described above, typically is corrected in rainbow trout through the inhibition of branchial HCO_3^- excretion (Cl^- uptake), with increased branchial H^+ excretion (Na^+ uptake) playing a minor role (Perry et al., 1987a). By contrast, rainbow trout appear to respond differently to a metabolic acidosis (incurred through HCl infusion); Na^+ uptake (H^+ excretion) is greatly enhanced but Cl^- uptake (HCO_3^-

excretion) falls only slightly (albeit significantly) (Goss and Wood, 1991). The net effect, increased net acid excretion, is similar to that observed with a respiratory acidosis, but the mechanism appears to be different. Given that Na^+ uptake is enhanced in subordinate trout (Sloman et al., 2003b, 2004), low social status would be predicted to be associated with an enhanced capacity to compensate for metabolic acid-base disturbances. This prediction remains to be tested.

Low social status results in a wide range of physiological changes many of which are detrimental (see above). Many of these physiological changes are mediated by the prolonged elevation of circulating cortisol levels such as a decrease in growth rate (Pottinger and Pickering, 2002; Overli et al., 1999a; Sloman et al., 1999a; reviewed by Sloman and Armstrong, 2002; Gilmour et al., 2005). Specific behaviours are also observed with low social status such as behavioural inhibition, including reduced activity, feeding and aggression (Abbott and Dill, 1985; McCarthy et al., 1992; Winberg et al., 1993b; Moutou et al., 1998; Overli et al., 1998). The chronic elevation of circulating cortisol also results in an overall poor condition of subordinate fish (reviewed in Gilmour et al., 2005) by depressing immune function (Abbott et al., 1985; Moutou et al., 1998; Pottinger and Pickering, 1992). Cortisol is also thought to have an effect on ionic/osmotic regulation in fish by altering the gill epithelia at the cellular and molecular levels (Laurent and Perry, 1990; Perry et al., 1992b), however this study found subordinate fish, although having higher circulating cortisol levels, were able to combat an acid-base disturbance similarly to control and dominant individuals. Despite responding appropriately to the stress of an acid-base disturbance subordinate individuals may suffer long-term effects on metabolism and growth.

This present study demonstrates that subordinate fish appear to compensate for the respiratory acidosis incurred by exposure to hypercapnia similarly to dominant and control

trout. Even though subordinate individuals appear unable to elevate net acid excretion during hypercapnia, these fish are able to regulate arterial blood pH as effectively as dominant and control trout, through the accumulation of HCO_3^-. Subtle differences in how this compensation occurs between subordinate and dominant individuals suggest there may be a cost to acid-base regulation.

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