Iranian Journal of Fisheries Sciences 21(1) 82-92 2022
DOI: 10.22092/ijfs.2022.125847

## Research Article

# Carbon dioxide anesthesia: A potential application to improve the air exposure duration of tilapia

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Received: March 2018 Accepted: January 2019

**Abstract**

The behavioral and physiological responses of tilapia to air exposure after pure saturated carbon dioxide ($CO_2$) anesthesia were analyzed, and a potential application of live tilapia dehydrated transport was evaluated by comparing it with the tricaine methanesulfonate (MS-222) anesthesia and no anesthesia. Tilapia displayed about 1 min of hyperactivity, and the induction time of anesthetized was about 3 min during $CO_2$ anesthesia, both the behavioral analysis and plasma cortisol levels showed that tilapia were more sensitive and stressed to the $CO_2$ anesthesia than MS-222 anesthesia. During air exposure, both anesthesia groups had good sedative effects, and the survival time of unanesthetized group, $CO_2$ anesthesia group and MS-222 anesthesia group were 331, 489 and 528 min respectively, and the survival time and survival rates of the anesthetized tilapia were significantly increased. Considering the efficacy, ease of use, as well as safety, the results of this study showed that $CO_2$ was an ideal anesthetic to improve air exposure duration of tilapia, and carbon dioxide anesthesia of tilapia potentially can be translated into an industrial short-time air exposure procedure.

**Keywords:** Carbon dioxide, Anesthesia, Tricaine Methanesulfonate, Tilapia, Behavioral and physiological response, Air exposure duration

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## Introduction

In recent years, tilapia (mainly *Oreochromis niloticus*) has become one of the largest single cultured fishery species in China, with a production of 1.65 million metric tons in 2013, of which 0.86 million tons of the production were used in processing frozen tilapia product (Fisheries Bureau, Ministry of Agriculture of The People's Republic of China, 2013). Currently, most farms are far from fish processing plants, tilapia are netted and placed in tank with an agitator for aeration, mixing with oxygen, and transported for about 2-5 hours to the processing plant. During transportation, water quality, physical damage and physiological problems were of great impacts on the mortality of tilapia (Colt and Kroeger, 2013). Fish were subjected to intense handling stress and might be injured or even die during the transport and handling procedures (Conte, 2004; Harmon, 2009). Air exposure is unavoidable for fish processing, however, air exposure for a short period of time (less than 10 min) is a significant stressor to fish (White *et al.*, 2008; Trushenski *et al.*, 2010). Air exposure also cause physical damage, which influence post-release behavior and survival of fish (Cook *et al.*, 2015). Although dehydrated (without large volume of water, only skin and gills were kept moist, and fish were exposed to air) transport or other handling procedure of live tilapia seems attractive, it is seldom used because of strong stress responses and high mortality. However, fish anesthetized properly could be transported without water, provided the skin and gills were kept moist under low temperature (Peter, 2008).

Anesthetics, such as tricaine methanesulfonate (MS-222), carbon dioxide ($CO_2$), benzocaine and phenoxyethanol have been used to sedate fish, lower metabolism and oxygen demand, and minimize stress responses and physical damages during handling procedures (Pramod *et al.*, 2010; Erikson, 2011; Vandergoot *et al.*, 2011; Cosenza, *et al.*, 2014; Oberg *et al.*, 2015). MS-222 is one of the most widely used anesthetic in fish, and it has high efficacy for anesthetizing fish (Carter *et al.*, 2011). However, a 21-day of withdrawal period is required by the US FDA for use on fish for food, and use of MS-222 should be restricted to Ictaluridae, Salmonidae, Esocidae and Percidae, and water temperature should not exceed 10°C. Therefore, use of MS-222 for anesthetizing food fish is often not practical. $CO_2$ is a food additive and widely used in the food industry, and it is also used as an anesthetic in fishery to immobilize fish before slaughter (Roth *et al.*, 2006; Erikson, 2008; Vandergoot *et al.*, 2011). Compared with other fish anesthetics, $CO_2$ is cheap and easy to use, in combination with the advantage that it leaves no toxic residues in the product. However, $CO_2$ anesthesia usually results in mild or strong stress response, so fish may be physically damaged or even die (Erikson, 2011; Seth *et al.*, 2013).

The objective of this study was to evaluate the effects of air exposure after carbon dioxide anesthesia on behavioral

and physiological responses of tilapia, by comparing it with the tricaine methanesulfonate anesthesia and no anesthesia. Results from this study have important implications for developing a short-time air exposure procedure for handling tilapia.

## Materials and methods

*Animals*

All the experiments were performed according to Guidelines of the European Union Council (86/609/EU) for the use of laboratory animals, and ethical permit approved by the Animal Ethics Committee of Guangxi University. Live tilapia (*Oreochromis niloticus*) ranging from 470-550g (mean±SE: 510±21g) were purchased in a local farm and transported to the laboratory, where they were acclimated for two days before the experiment began in continuously aerated 50L tanks, with a stocking density of 20 fish per tank (15±1°C, pH 6.8-7.2, dissolved oxygen levels 5.9-7.3 mg $L^{-1}$).

*Behavioral analysis*

Behavior of the two fish groups, the $CO_2$ and MS-222 anesthesia tilapia, was recorded using a video camera and visually observed. The indicator of consciousness was assessed according to the method of Roth *et al.* (2003). Behavioral indicators at different stages of anesthesia are described in Table 1: (such as Stage 3: No swimming activity, problems of ventilation of operculum, total loss of equilibrium - fish turn over). A total of 16 tilapia were observed for each group. Each experiment was performed in quadruplicate.

**Table 1: Behavioral responses and induction time (seconds) of tilapia exposure to $CO_2$ and MS-222, respectively.**

| Stage | Behavioral responses | Induction time (s) | |
|---|---|---|---|
| | | **$CO_2$ anesthesia** | **MS-222 anesthesia** |
| 0 (Normal) | Normal<br>Then appear agitated, exhibit jumping behavior. | 0-30<br>30-90 | 0-10<br>10-30 |
| 1 (Light sedation) | Reduced swimming activity, had problems with equilibrium, but with normal ventilation of operculum. | $117\pm23^{a,x}$ | $34\pm5^{a,y}$ |
| 2 (Narcosis) | Weak swimming activity, slow and long ventilation rate, equilibrium loss with efforts to right. | $135\pm17^{a,x}$ | $157\pm9^{b,y}$ |
| 3 (Deep narcosis) | No swimming activity, problems of ventilation of operculum and total loss of equilibrium - fish turn over. | $171\pm15^{b,x}$ | $293\pm16^{c,y}$ |

$^{a, b, c}$ Data in column with the same superscripts are not significantly different while data with different superscripts are significantly different ($p$<0.05). $^{x, y}$ Data between $CO_2$ and MS-222 anesthesia with different superscripts are significantly different ($p$<0.05).

*Carbon dioxide anesthesia experiment*

Forty-five fish were randomly divided into three groups:

Group 1 ($CO_2$ anesthesia group), $CO_2$-saturated water (pH 5.60-5.65) was prepared via flushing with pure $CO_2$ gas during this procedure. Fish ($n$=16) were

exposed to $CO_2$-saturated water with the density of 2 fish $L^{-1}$ at 15±1°C. Each experiment was performed in quadruplicate.

Group 2 (MS-222 anesthesia group), Fish ($n$=16) were exposed to 100 mg $L^{-1}$ of MS-222 buffered with $NaHCO_3$ (200 mg $L^{-1}$) in aerated water, with the density of 2 fish $L^{-1}$ at 15±1°C. Each experiment was performed in quadruplicate. MS-222 anesthesia was used as a positive control.

As long as the fish of the two groups reached the third stage of anesthesia, they were immediately netted from the anesthetized tank, then placed in a temperature-controlled (15±1°C) box for air exposure experiment. The choice of doses, temperature and time used was based on our previous study (Fan *et al*., 2014).

Group 3 (control/unanesthetized group), Control fish ($n$=13) were put in the basket directly without being anesthetized.

*Air exposure experiment*

During the air exposure experiment, fish were placed in a basket and then the basket was put in a constant-temperature box (15±1°C), and fish were sprayed constant-temperature water (15±1°C) every 10 min to kept moist of the skin and gills. Fish was considered to be dead when its respiratory movement ceased (Roth *et al.*, 2003). Fish survival time and survival rates at different times were the metric used to evaluate each treatment.

*Determination of blood variables*

Blood samples (0.5 mL $sample^{-1}$) were collected by using heparinised syringes as soon as fish were anesthetized before air exposure and after 450 min of air exposure (for the $CO_2$ and MS-222 anesthesia group), and after 300 min of air exposure (for the control group). Whole blood was spun and plasma was collected. Subsequently, a clinical blood analyzer (TBA-120FR, Toshiba medical systems Co., Japan) was used to analyze lactate (Lac) and glucose (Glu). Plasma cortisol was analyzed with the radioimmunoassay technique (DPC Immulite 2000, DPC Cirrus Co., USA). Each experiment was performed in triplicate. The data of MS-222 anesthesia before air exposure in this study were also used as the baseline data.

*Statistical analysis*

SPSS statistics 17.0 (SPSS Inc., Chicago, USA) was used for analyzing the data. Analysis of Variance (ANOVA) was used to determine the significant differences ($p$<0.05) among the treatments.

## Results

*Behavioral responses*

Initially after anesthetic exposure, tilapia in both groups swam around calmly, then after tens of seconds, they appeared agitated by exhibition jumping behavior (hyperactivity) and attempted to escape (Table 1). The hyperactivity was more intense in the $CO_2$ anesthesia experiment. After the anesthetics began to take effect, fish reached the second stage of light sedation (narcosis). In the

third stage, fish were in deep narcosis, and they had reduced ventilation of operculum and exhibited total loss of equilibrium. At the third stage, tilapia were immediately netted from the anesthetized tank, and placed in a temperature-controlled box for the air exposure experiment.

In the $CO_2$ anesthesia experiment, although tilapia displayed a brief and violent struggle, there were markedly faster induction times compared with MS-222 anesthesia experiment (171 s vs. 293 s). During air exposure, there were no jumping behavior in both the $CO_2$ and MS-222 anesthesia group fish, while the control group fish were jumping for nearly 20 min, then they were exhausted.

*Blood parameters and plasma cortisol*

As shown in Figure 1, the $CO_2$ anesthesia experiment induced a significant increase in plasma cortisol levels as compared with MS-222 anesthesia experiment. Because MS-222 is a very mild anesthetic, and it's widely used for anesthetizing fish to get the baseline data of fish physiology (Carter *et al.*, 2011), the data of MS-222 anesthesia before air exposure in this study were also used as the baseline data. After 300 min (for the control group) and 450 min (for the $CO_2$ and MS-222 anesthesia groups) of air exposure, the plasma cortisol levels in all groups increased up to 4-5 times higher than the baseline data. The plasma cortisol level in $CO_2$ anesthesia experiment was higher than those of the MS-222 anesthesia and control treatments. The blood plasma glucose level in $CO_2$ anesthesia treatment was significantly higher than that of the MS-222 anesthesia treatment, while lactate level in $CO_2$ anesthesia treatment was significantly lower than that of the MS-222 anesthesia treatment. After air exposure, as expected, the plasma glucose levels in all groups dropped sharply, while lactate levels rose rapidly at the same time.

*Survival rates and survival time during air exposure*

Survival rate of the control group after 280 min of air exposure was 100% (Table 2), and then began decreasing as air exposure time continued, with the bulk of mortalities occurring at 280-400 min of air exposure. No mortality was observed in the $CO_2$ and MS-222 anesthesia groups within 450 min of air exposure. Most of the fish died between 480-500 min (for $CO_2$ anesthesia group) and 480-600 min (for MS-222 anesthesia group) of air exposure. The survival time of the control group, $CO_2$ anesthesia group and MS-222 anesthesia group were 331, 489 and 528 min, respectively. Survival rates and survival time in the control group were significantly lower than in the $CO_2$ and MS-222 anesthesia groups. The MS-222 anesthesia group had the highest survival rate and survival time.

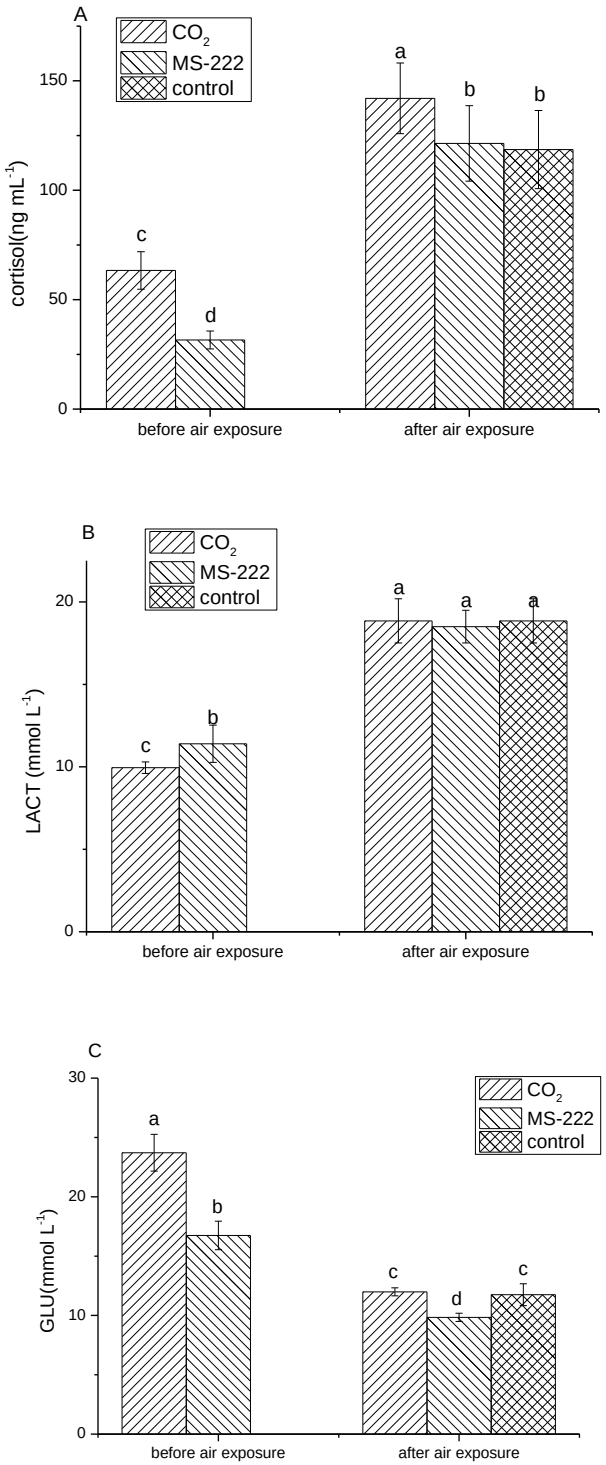


**Figure 1: Plasma cortisol and blood parameters in tilapia at time of 0 min (before air exposure) and after 300 min (for the control group) and 450 min (for the $CO_2$ and MS-222 anesthesia groups) of air exposure.**

**Table 2: Survival rates at different times and survival time of the three groups of tilapia during air exposure (N=10).**

| Air exposure time (min) | Survival rate (%) | | |
|---|---|---|---|
| | $CO_2$ anesthesia group | MS-222 anesthesia group | Control group |
| 280 | 100 | 100 | 100 |
| 300 | 100 | 100 | 80 |
| 350 | 100 | 100 | 40 |
| 400 | 100 | 100 | 0 |
| 450 | 100 | 100 | 0 |
| 480 | 80 | 100 | 0 |
| 500 | 10 | 70 | 0 |
| 550 | 0 | 40 | 0 |
| 600 | 0 | 0 | 0 |
| Survival time (min) | $489\pm9^a$ | $528\pm32^b$ | $331\pm25^c$ |

a, b, c Data in row with different superscripts are significantly different ($p$<0.05).

## Discussion

Behavioral analysis showed that tilapia were more sensitive and stressed to $CO_2$ anesthesia than MS-222. MS-222 is generally considered to be effective for anesthetizing fish, and it has little side effect on stress when properly used (Carter *et al.*, 2011). MS-222 is absorbed rapidly through gills and has direct actions on the central nervous system, and exerts it sedative effect by preventing generation and conduction of nervous impulse (Carter *et al.*, 2011). In this study, tilapia displayed a very short and weak struggling behavior, and then came into the light sedation stage during MS-222 anesthesia, which proved that MS-222 was effective for anaesthetizing tilapia. The effectiveness of $CO_2$ is due to causing hypercapnia, which can depress the central nervous system, resulting in the loss of consciousness and voluntary motor action (Sanderson and Hubert, 2007). When anesthetizing fish with $CO_2$, there is a hypothesis that supplemental oxygen (i.e. hyperoxic carbon dioxide anesthesia) may reduce stress during $CO_2$ anesthesia (Kohler *et al.*, 1999). However, some studies (Sandblom *et al.*, 2013) have examined the physiological responses to hyperoxic carbon dioxide anesthesia in fish, and found that the increase in plasma cortisol was similar with the pure $CO_2$ anesthesia, which suggested that supplemental oxygen did not markedly reduce stress. Therefore, pure saturated $CO_2$ was deliberately chosen to anesthetize the fish rapidly for this study. In this study, tilapia displayed about 1 min of hyperactivity, with an induction time of about 3 min during $CO_2$ anesthesia. These results were similar to those of using $CO_2$ anesthesia of Arctic char (Sandblom *et al.*, 2013; Seth *et al.*, 2013), common snook (*Centropomus undecimalis*), and Florida pompano (*Trachinotus carolinus*) (Oberg *et al.*, 2015).

Plasma cortisol level is a sensitive indicator of primary stress response (Bonga, 1997). Therefore, measurements of cortisol are common in the assessments of stress response in fish (Foo and Lam, 1993; Sandblom *et al.*, 2013; Seth *et al.*, 2013). In this study, plasma cortisol levels increased significantly after $CO_2$ anesthesia, and

the cortisol level in $CO_2$ anesthesia treatment was twice that of the MS-222 anesthesia, which means that $CO_2$ anesthesia treatment tilapia specimens were more stressed as compared with MS-222 anesthesia treatment. Plasma cortisol levels coincided with the behavioral analysis results. Stress during anesthesia process may be unavoidable. However, compared with other more severe handling stress, for example, the cortisol level was over 100 ng $mL^{-1}$ when tilapia were confined (Vijayan *et al.*, 1997). Therefore, $CO_2$ anesthesia in the present study led to a medium stress response as compared with the very mild MS-222 anesthesia.

An increase in plasma glucose level is a typical secondary stress response resulting from activation of glycogenolysis and/or glyconeogenesis by the action of cortisol (Polakof *et al*., 2012). As usual, elevated plasma cortisol level is bounded with the increase of plasma glucose and lactate levels. In the case of our $CO_2$ and MS-222 anesthesia experiment, the relationship between changes in the plasma cortisol and plasma glucose was quite obvious. $CO_2$ anesthesia induced a strong release of cortisol, and the maximum plasma cortisol level corresponded to the maximum plasma glucose. However, lactate level in $CO_2$ anesthesia treatment was significantly lower compared with that in the MS-222 anesthesia treatment; perhaps as the induction time of $CO_2$ anesthesia was shorter than MS-222 anesthesia (3 min vs. 5 min), and so the accumulation of lactate was less.

As expected, air exposure had great affects on tilapia. Plasma cortisol levels increased sharply after air exposure, so air exposure was a severe stressor to tilapia. During air exposure, there was no oxygen available and aerobic metabolism was shifted to anaerobic metabolism. Anaerobic glycolysis provides energy by breakdown of glucose without the need of oxygen, and lactate is the by-product of anaerobic metabolism (Polakof *et al.*, 2012). Therefore, the plasma glucose levels dropped and lactate levels increased sharply after air exposure. After 300 min (for the control group) and 450 min (for the $CO_2$ and MS-222 anesthesia groups) of air exposure, the plasma lactate levels in all the groups accumulated to 18.5-18.9 mmol $L^{-1}$, and energy depletion made fish difficult to stay alive.

Two remarkable phenomena were observed that both the survival rates and survival time of the anesthetized tilapia were significantly increased during air exposure. Anesthesia could sedate fish and lower the metabolism (Carter *et al.*, 2011). For example, at the beginning of air exposure, the control fish were jumping until exhaustion, while anesthetized fish were sedate. Therefore, anesthesia might have prolonged the survival time of tilapia during air exposure. In this study, both the MS-222 anesthesia and the $CO_2$ anesthesia had exactly good effects, and survival time of their groups was 197 min and 158 min longer than that of the control group, respectively. It would be convenient for the tilapia processing plant if the tilapia were sedate when it is exposed to air for

a short time (about 100-400 min) during the transport and processing procedure. In the present study both anesthesia groups were under good sedative effects that prolonged the survival time during air exposure. Therefore, it seemed that anesthesia would be suitable for improving the air exposure duration of tilapia and would be useful in the tilapia processing plant.

Considering the efficacy, ease of use, as well as safety, $CO_2$ was an ideal anesthetic for tilapia. Pure saturated $CO_2$ anesthesia only led to a medium stress response of tilapia. $CO_2$ anesthetized tilapia was under good sedative effects during air exposure, the air exposure duration was improved and the survival time was prolonged to 489 min. Therefore, a short-time air exposure procedure could be practical when tilapia was properly anesthetized by $CO_2$.

**Acknowledgments**

This work was funded by "National Natural Science Foundation of China (No. 31660448)", "Guangxi Natural Science Foundation (No. 2016GXNSFAA380290 and 2016GXNSFEA380003)", "The Nanning City Science and Technology Promotion Project (No. 20142306)" and "BaGui Scholars Program of Food Biotechnology".

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