Effect of semen cryopreservation with cucumber (Cucumis sativus) fruit juice (CJ) fortified- Extender on milt quality, viability and oxidative enzyme activity of Clarias gariepinus (Burchell, 1822)

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Abstract
The present study investigated the quality, viability and oxidative enzyme activity of catfish semen fortified with cucumber fruit juice. The highest milt motility duration time of 66.6 seconds occurred among 20% CJ and stored for 4 days. The lowest duration time of 50.3 s was recorded among the control group of milt stored for 2 days. The motility rate differed significantly between the control and CJ. The highest rate of 99.21% recorded in control milt on day 4 compared with least rate of 81.95% recorded among 15% CJ on day 1. The highest milt fertility rate of 75.35% recorded in 10% CJ on day 2 compared with the least rate of 56.63% in control on day 1. The least hatchability rate of 64.21% recorded in control day 3 compared with the highest value of 74.73% in 10% CJ on day 2. The highest milt survival rate of 99.46% was recorded among the control group on day 2 which compared with the least survival rate of 87.66% recorded on day 1 of 10, 15 and 20% CJ. There was a significant difference (P< 0.05) between the CAT control and treatments of CJ. The lowest value of 0.25 μmolem⁻¹ mgprotein⁻¹ recorded in control on day 1 compared with the highest value of 0.34μmolem⁻¹ mgprotein⁻¹ in 15% CJ on day 4. There was no significant difference in SOD activity between the control and CJ. Recorded values ranged between 0.026-0.56 μmolem⁻¹ mgprotein⁻¹ in 15% on day 2 and 10% day 1 respectively. There was no significant difference in LPO activity between the control and CJ. However, a range of 0.023-0.130 mMoleTbarsmm⁻¹ day⁻¹ recorded on day 1 in 10% control respectively. 20 and 10% Cucumber fruit juice showed greater values in motility duration and fertility rate than control at respective storage periods for 4 and 2 days in Clarias gariepinus semen, and could in combination with DMSO improve its cryopreservation at short periods.

Keywords: Cryopreservation, fish semen, oxidative enzyme activity, motility duration, semen quality

Introduction
Cryopreservation is the use of low temperatures to preserve structurally intact living cells such as milt of fish in diluents comprising of the extender and cryoprotectant chemicals. The extender increases the volume of the semen and conserves its endogenous energy while the cryoprotecting chemicals such as dimethyl sulfoxide DMSO limit cell injury resulting from freezing and thawing but more often introduces toxicity concerns [19]. Stock protection from disease, environmental and natural hazards is limited by cryopreservation and could preserve endangered species in perpetuity [2,1]. It provides ease for transport, suitable supply of sperms in hatcheries and laboratories and availability in selective breeding and gene transfers [18]. Reported that coconut water with suitable salts of potassium, sodium and sugars provide good extender chemical for milt cryopreservation of Clarias gariepinus. The effectiveness of cryopreservation success in recent times is the toxicity concerns posed by cryoprotecting chemicals which have been implicated in oxidative stress of cryopreserved semen. Fish semen generally contains limited antioxidant enzymes and requires nontoxic and environmental friendly natural substances rich antioxidants such as in fruit juice. Cucumber Cucumis sativus fruit juice and seed extracts have been reported to contain high level antioxidant and antibacterial properties [23] which may be suitable to limit oxidative stress posed by DMSO in milt cryopreservation. There is scarcity of data on fish milt quality and antioxidant activity and growth of larvae obtained from fertilized eggs by cryopreserved spermatozoa. The aim of this...
Research therefore is to determine the effect of semen Cryopreservation with cucumber fruit juice fortified- Extender on milt quality, viability, anti-oxidative enzyme activity and the growth of *Clarias gariepinus* larvae fertilized by cryopreserved milt. The objectives of this study will be to: [1] Determine the effects of adding cucumber fruit juice to coconut-water extender on cryopreserved milt quality and viability of *Clarias gariepinus* [2]. Compare the anti-oxidative enzymes of cryopreserved fish milt in extender- fortified in cucumber fruit juice with DMSO as control.

**Materials and methods**

**The study area**

The study was conducted at the Faculty Research Farm, Fisheries Unit Faculty of Agriculture, Enugu State University of Science and Technology, Agbani, Enugu. This site is located at latitude 07°48.20'N; Longitude 06°38.78’E. It has an annual rainfall of 200mm and daily temperature range from 20 °C to 35 °C with an average of 26.7 °C.

Collection of experimental fish, semen and eggs

Preparation of extender, cryoprotectant and fruit juice from cucumber

Matured high quality brood stock was obtained from a reputable farm in Enugu. The male and female shall be sacrificed and stripped to obtain milt and egg respectively.

Collection of experimental fish, semen and eggs

Matured high quality brood stock was obtained from a local farm in Enugu Nigeria. The male and female shall be sacrificed and stripped to obtain milt and egg respectively.

Coconut water obtained from endosperm of coconut fruit was procured from a local market also in Enugu metropolis.

Diluents (mixture of extender and cryoprotectant) shall be prepared and stored in the refrigerator at 4 °C for 24hrs before use [18, 20]. 2% of DMSO was prepared and added as cryoprotectant. Thereafter milt was diluted at 1:20 with diluent and equilibrated for 30 minutes [26] before freezing in programmable freezer for 4 days. Sperm quality was assessed daily

**Sperm quality**

Sperm motility and duration was estimated subjectively under the microscope following the method described by [3].

**Sperm viability**

Cryopreserved sperms straws at the end of freezing, was thawed at 37 °C for 30s in water bath, and cut open from the sealed end to release sperms used to fertilize pooled ripe eggs from gravid brood females. Fertilized eggs incubated for 30h at appropriate temperature and pH of water. Fertilization, hatchability and survival rate of larvae was used to assess the viability of the milt.

Fertilization rate (%) = Number of egg cells hatched × 100
Total number of egg cells counted

Hatchability rate (%) = Number of eggs hatched × 100
Total number of eggs in a batch

Survival rate (%) = Number of hatching alive to larval stage × 100
Total number of hatchlings

**Anti-oxidative enzyme activity**

The catalase (CAT) in the semen was determined according to the method of [24] which involved H$_2$O$_2$ breakdown, and was measured spectrophotometrically at 240 nm. Enzyme activity will be expressed as mini moles of H$_2$O$_2$ decomposed min/L mg/L protein.

Superoxide dismutase (SOD) activity was determined using the method of [14], based on the oxidation of epinephrine-adenochrome transition by the enzymes. Superoxide dismutase activity was assed spectrophotometrically at 420 nm and expressed as the amount of enzyme mg/L of protein required to give 50% inhibition of epinephrine auto-oxidation. Lipid peroxidase (LPO) was determined by estimation of thiobarbituric acid reactive substances (TBARS), according to [22]. TBARS concentration was measured spectrophotometrically at 535 nm at molar extinction coefficient of 156 nm cm/L. Enzyme activity was expressed in mini moles of TBARS mg/L protein.

**Results**

The results of milt motility duration, motility rate, fertility rate, hatchability rate, survival and oxidative stress is given in table 1 below.

**Table 1:** Milt motility duration, motility rate, fertility rate, hatchability rate, survival and oxidative stress using Varying levels of Cucumber Juice for 4 days

| Parameters CJ in Days | Mean   | Std. Error (±) |
|-----------------------|--------|----------------|
| control day 1         | 50.8000| .05774         |
| control day 2         | 50.3000| .05774         |
| control day 3         | 51.2600| .05774         |
| control day 4         | 51.6000| .05774         |
| 5% CJ day 1           | 53.3400| .05774         |
| 5% CJ day 2           | 52.8033| .05487         |
| 5% CJ day 3           | 53.8067| .05207         |
| 5% CJ day 4           | 54.5200| .23180         |
| 10% CJ day 1          | 55.8233| .03930         |
| 10% CJ day 2          | 55.3200| .05774         |
| 10% CJ day 3          | 56.3800| .05774         |
| 10% day 4             | 59.3300| .05774         |
| 15% day 1             | 56.7233| .79386         |
| 15% CJ day 2          | 56.1433| .79386         |
| 15% CJ day 3          | 57.2100| .81206         |
| 15% CJ day 4          | 64.2433| 2.50683        |
| 20% CJ day 1          | 55.8800| .05774         |
| 20% CJ day 2          | 55.3000| .05774         |
| 20% CJ day 3          | 56.3000| .05774         |
| 20% CJ day 4          | 66.6000| .05774         |
| Total                 | 55.6842| .53275         |
|                | control day 1 | control day 2 | control day 3 | control day 4 | 5% CJ day 1 | 5% CJ day 2 | 5% CJ day 3 | 5% day 4 | 10% CJ day 1 | 10% CJ day 2 | 10% CJ day 3 | 10% day 4 | 15% day 1 | 15% CJ day 2 | 15% CJ day 3 | 15% CJ day 4 | 20% CJ day 1 | 20% CJ day 2 | 20% CJ day 3 | 20% CJ day 4 | 20% day 4 | Total |
|----------------|---------------|---------------|---------------|---------------|-------------|-------------|-------------|---------|--------------|--------------|--------------|-----------|-----------|--------------|-------------|-------------|--------------|--------------|--------------|--------------|---------|--------|
| **Motility rate (%)** | 96.4100       | 96.9100       | 98.4067       | 99.2100       | 91.5900     | 92.0700     | 93.4900     | 94.2500 | 86.5000      | 87.2200      | 88.5700      | 89.2900   | 81.9500   | 82.3800      | 83.4100     | 84.3300     | 86.4100      | 87.2100      | 88.5100      | 89.3400      | 89.8728  | 66.739 |
| **Fertility rate (%)** | 56.6300       | 68.4667       | 64.7000       | 60.0100       | 69.4600     | 71.9300     | 67.9433     | 62.0600 | 62.2900      | 75.3500      | 71.2000      | 65.0700   | 62.1967   | 75.3000      | 71.2000     | 65.3000     | 62.1967      | 70.3000      | 65.0700      | 62.1967      | 66.5798  | .72530 |
| **hatchability_rate (%)** | 57.9700       | 67.9400       | 64.2100       | 59.3700       | 60.8700     | 71.3400     | 67.4200     | 62.3400 | 63.7700      | 74.7300      | 70.8300      | 65.3100   | 63.7100   | 74.7100      | 70.8100     | 65.3100     | 63.7100      | 74.7100      | 70.8100      | 65.3100      | 66.7590  | .64346 |
| **Survival rate (%)** | 97.4667       | 99.4667       | 99.1967       | 98.5667       | 92.5967     | 99.1967     | 98.5667     | 92.5967 | 63.7700      | 74.7300      | 70.8300      | 65.3100   | 63.7100   | 74.7100      | 70.8100     | 65.3100     | 63.7100      | 74.7100      | 70.8100      | 65.3100      | 66.7590  | .63333 |
| CAT (µmole:mm-1mgprotein⁻¹) | 5% CJ day 2 | 94.4967 | .03333 |
|-----------------------------|--------------|---------|---------|
| 5% CJ day 3                 | 94.2367      | .03333 |
| 5% day 4                    | 93.6467      | .03333 |
| 10% CJ day 1                | 87.6933      | .03333 |
| 10% CJ day 2                | 89.5267      | .03333 |
| 10% CJ day 3                | 89.2867      | .03333 |
| 10% day 4                   | 88.7167      | .03333 |
| 15% CJ day 1                | 87.6667      | .03333 |
| 15% CJ day 2                | 89.4667      | .03333 |
| 15% CJ day 3                | 89.2867      | .03333 |
| 20% CJ day 1                | 87.6667      | .03333 |
| 20% CJ day 2                | 89.4667      | .03333 |
| 20% CJ day 3                | 89.2867      | .03333 |
| 20% CJ day 4                | 88.6667      | .03333 |
| Total                       | 91.7515      | .52402 |

| SOD (Umole:mm-1mgprotein⁻¹) | control day 1 | .2904 | .00007 |
|-----------------------------|----------------|------|--------|
| control day 2               | .2927          | .00033 |
| control day 3               | .2947          | .00033 |
| control day 4               | .2957          | .00033 |
| 5% CJ day 1                 | .3052          | .00032 |
| 5% CJ day 2                 | .3070          | .00032 |
| 5% CJ day 3                 | .3098          | .00003 |
| 5% day 4                    | .3099          | .00003 |
| 10% CJ day 1                | .3194          | .00003 |
| 10% CJ day 2                | .3213          | .00003 |
| 10% CJ day 3                | .3235          | .00003 |
| 10% day 4                   | .3246          | .00003 |
| 15% CJ day 1                | .3339          | .00003 |
| 15% CJ day 2                | .3359          | .00003 |
| 15% CJ day 3                | .3382          | .00003 |
| 15% CJ day 4                | .3394          | .00003 |
| 20% CJ day 1                | .3197          | .00033 |
| 20% CJ day 2                | .3217          | .00033 |
| 20% CJ day 3                | .3237          | .00033 |
| 20% CJ day 4                | .3249          | .00003 |
| Total                       | .3166          | .00194 |

| LPO (mMole:Tbars:mm-1protein⁻¹) | control day 1 | .0030 | .00000 |
|---------------------------------|----------------|------|--------|
| control day 2                   | .0040          | .00000 |
| control day 3                   | .0070          | .00000 |
| control day 4                   | .0090          | .00000 |
| 5% CJ day 1                     | .0030          | .00058 |
| 5% CJ day 2                     | .0040          | .00058 |
| 5% CJ day 3                     | .0070          | .00058 |
| 5% day 4                        | .0090          | .00058 |
| 10% CJ day 1                    | .0023          | .00033 |
| 10% CJ day 2                    | .0033          | .00033 |
Milt motility duration
The highest milt motility duration time of 66.6 seconds occurred among 20% CJ, stored for 4 days. The lowest duration time of 50.3 s was recorded among the control group of milt stored for 2 days (Figure 1).

| Treatment | Day   | Motility Duration | p-value |
|-----------|-------|-------------------|---------|
| 10% CJ    | day 3 | .0073             | .00033  |
| 10%       | day 4 | .0133             | .00333  |
| 15%       | day 1 | .0030             | .00058  |
| 15% CJ    | day 2 | .0050             | .00058  |
| 15%       | day 3 | .0070             | .00058  |
| 15% CJ    | day 4 | .0133             | .00333  |
| 20%       | day 1 | .0030             | .00058  |
| 20% CJ    | day 2 | .0040             | .00058  |
| 20%       | day 3 | .0070             | .00058  |
| 20% CJ    | day 4 | .0090             | .00058  |
| Total     |       | .0062             | .00047  |

Fig 1: Mean of motility duration

Fig 2: Mean of motility rate
Motility rate
The motility rate differed significantly between the control and CJ. The highest rate of 99.21% recorded in control milt on day 4 compared with least rate of 81.95% recorded among 15% CJ on day 1 (Fig. 2).

Fertility rate
The highest milt fertility rate of 75.35% recorded in 10% CJ on day 2 compared with the least rate of 56.63% in control on day 1 (Fig. 3).

Hatchability rate
The least hatchability rate of 64.21% recorded in control day 3 compared with the highest value of 74.73% in 10% CJ on day 2 (Fig. 4).
Milt survival rate
The highest milt survival rate of 99.46% was recorded among the control group on day 2 which compared with the least survival rate of 87.66% recorded on day 1 of 10, 15 and 20% CJ (Fig. 5).

Catalase activity
There was a significant difference ($P < 0.05$) between the control and treatments of CJ. The lowest value of 0.25 µmolemm$^{-1}$mgprotein$^{-1}$ recorded in control on day 1 compared with the highest value of 0.34 µmolemm$^{-1}$mgprotein$^{-1}$ in 15% CJ on day 4 (Fig. 6).

SOD Activity
There was no significant difference in SOD activity between the control and CJ. Recorded values ranged between 0.026-0.56 Umole$^{-1}$mgprotein$^{-1}$ in 15% on day 2 and 10% day 1 respectively (Table 1).

LPO
There was no significant difference in LPO activity between the control and CJ. However, a range of 0.023-0.130 mMoleTbarsmm$^{-1}$protein$^{-1}$ was recorded on day 1 in 10% and control respectively (Table 1).

Discussion
The uses of fruit juices as cryopreservants have been reported to be suitable for the cryopreservation of major fish species (Heinstra et al., 2005; Horvath and Ubanyi, 2009) [12, 13]. In addition, many cryopreservation studies revealed that fruit juices resulted in higher fertilization and hatching rate
compared to artificial cryopreservars. Van Vuren and Steyn (2017) [25] noted however that the level, type, concentration, temperature, and exposure period of the fruit juice function to evaluate the quality and viability of the milt. Fruit antioxidants include carotenoids, vitamins, phenolic compounds and flavonoids and have proved to function as singlet and triplet oxygen quenchers, free radical scavengers and peroxide decomposers (Anghel et al., 2010; Daramola et al., 2016; [6, 9, 10]. Preservation of fish milt has become an uphill task due to the fact that most artificial cryo-protectants in use have oxidative impact on fish milt spermatozoa. Cucumber (Cucumis sativus) and orange (Citrus sinensis) are fruit-rich natural antioxidants renowned for high concentrations of these vitamins and other antioxidants (Cutis et al., 2013; Reda et al., 2016; Okiyele et al 2019) [8, 21, 19]. Cryopreserved catfish semen fertilizes more number of eggs than natural (Agarwal, 2005; Agarwal, 2011) [4, 5]. Dilution with extenders in cryopreservation of fish semen increases the volume of semen, so that it can be used for multiple inseminate (Agarwal, 2011) [5]. It was noted that in several trials of experiment, cryopreserved semen resulted in significantly higher fertilization and hatchability percentage than freshly extracted semen. (Kovacs and Urbanyi, 2010; Ezike et al., 2019) [14, 15]. The CAT-SOD system of enzyme in the semen may efficiently have removed reactive oxygen species ROS by a trigger from fruit antioxidants (Krzyzosiak et al., 2000) [15] produced during storage thus limited the elicitation of LPO. Therefore the use of cucumber fruit juice could advance cryopreservation of fish milt and in particular Clarias gariepinus (Adeyemo et al 2007; Boryshpolets et al., 2011; Liu et al., 2015) [3, 7, 10].

Conclusion
Although the control indicated higher milt motility, hatchability and survival rates than CJ. 20 and 10% Cucumber fruit juice showed greater values in motility duration and fertility rate than control at respective storage periods for 4 and 2 days in Clarias gariepinus semen, and could in combination with DMSO improve its cryopreservation at short periods.

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