INFLUENCE OF HORMONAL INDUCEMENT ON THE
SPERMATOLOGICAL QUALITIES OF CYPRINUS CARPIO

Thesis submitted in part fulfillment of the requirement for the
Degree of Master of Fisheries Science in Aquaculture to the
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ABSTRACT

Title : Influence of hormonal inducement on the spermatological qualities of *Cyprinus carpio*

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Changes on the spermatological qualities of *Cyprinus carpio* milt induced with three synthetic hormones at two different doses during short term preservation were assessed in the present study. Selected brooders of *C. carpio* (600±70g ABW) were induced with three hormones, *viz.*, Ovatide, Wova-FH and Ovaprim at doses of 0.3 and 0.6 ml/kg BW. Milt collected from naturally matured *C.carpio* was kept as Control. The milt from all the treatments were milky white in colour. The volume and pH of milt ranged from 0.8 to 1.3 ml and 7.2 to 7.4, respectively.

The milt was diluted 40 times with 0.85% physiological saline solution as extender for analysis. The mean sperm density of all samples were 3.2×10⁹ cells/ml. The highest mean sperm density of 4.3 ± 0.25×10⁹ Cells/ml was found with milt collected from males induced with Ovaprim at 0.3 ml/kg BW (Op₁). The lowest mean sperm density of 1.2×10⁹ cells/ml was noted when Ovaprim was given at the dose of 0.6ml/kg BW. There was significant variation (P>0.01)
among the treatments for the spermatozoa density that was caused due to different doses of hormones administered.

The diluted milt was short term preserved at 4°C in the refrigerator for 5 days. The spermatological parameters such as motility duration, motility score, motility pattern, percentage of live cells and percentage reduction in the motility performance were observed and calculated at an interval of 24 h.

Milt collected from non-induced fish (Control) had initial mean motility duration of 134±20.8 s. The highest motility duration was 178 ± 1.75 s for Ovatide 0.6ml/kg BW and lowest motility duration 123±16 s was seen when Ovatide was given at 0.6ml/kg BW. There were significant differences between Control and Ovaprim (0.6 ml/kg BW) induced and in motility duration of the spermatozoa collected from Control and Wova-FH (0.6 ml/kg BW) induced spermatozoa. The values were statistically significant at (P>0.01).

Among the three hormones tried, Ovatide (0.6 ml/kg BW) induced spermatozoa gave the best result in terms of motility duration and there was least reduction percentage (178±1.75 s and 19.0±4.97% respectively) was noticed. There were significant differences in the motility duration between Ovatide at 0.3 ml/kg BW and Ovatide at 0.6 ml/kg BW (P>0.01). The percentage of live cells was also higher at the end of the storage periods when Ovatide was used for induction at 0.6 ml/kg BW.

Spermatozoa collected from male *C. carpio* induced with Wova-FH at 0.6 ml/kg BW showed moderate motility duration (176 ±1.75 s). The values were statistically significant between the two doses of Wova-FH and between Control and Wova-FH at 0.6 ml/kg BW (P>0.01). The mean motility reduction percentage was 23.4±7.2% and 21.2±6.61% when the
milt samples where induced with Wova-FH at 0.3 and 0.6 ml/kg BW respectively. There was no significant difference in the reduction rate of motility duration of spermatozoa collected from Wova-FH induced male *C. carpio*.

Ovaprim gave the poor results when given at a dose of 0.3 and 0.6 ml/kg BW. The initial mean motility duration of the spermatozoa was 110±6.23 s and 153±6.12 s when induced with Ovaprim at 0.3 and 0.6 ml/kg BW respectively. However, the values were statistically significant between the two doses of Ovaprim and between Control and Ovaprim 0.6 ml/kg BW (P>0.01). The mean motility reduction percentage was the highest (26.6±9.3%) when Ovaprim was given at 0.3 ml/kg BW while compared with other treatments. However, this was not statistically significant.
## CONTENTS

<table>
<thead>
<tr>
<th>Chapter No.</th>
<th>Title</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>INTRODUCTION</td>
<td>1-6</td>
</tr>
<tr>
<td>2.</td>
<td>REVIEW OF LITERATURE</td>
<td>7-23</td>
</tr>
<tr>
<td></td>
<td>2.1 Hormones</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>2.2 Induced breeding of fishes</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>2.3 Inducement with natural hormone</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>2.3.1 Pituitary extracts</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>2.3.2 Human Chorionic Gonadotropin (HCG)</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>2.4 Inducement with synthetic hormones</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>2.4.1 Ovatide</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>2.4.2 Wova-FH</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>2.4.3 Ovaprim</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>2.5 Spermatological parameters of fish spermatozoa</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>2.5.1 Colour and viscosity</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>2.5.2 Sperm count</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>2.5.3 Motility duration</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>2.5.4 Milt pH</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>2.6 Factors affecting the spermatological parameters of fish</td>
<td>19</td>
</tr>
<tr>
<td>Chapter No.</td>
<td>Title</td>
<td>Page No.</td>
</tr>
<tr>
<td>------------</td>
<td>--------------------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>2.6.1</td>
<td>Seasonal changes</td>
<td>20</td>
</tr>
<tr>
<td>2.6.2</td>
<td>Temperature</td>
<td>20</td>
</tr>
<tr>
<td>2.6.3</td>
<td>Age of broodstock</td>
<td>21</td>
</tr>
<tr>
<td>2.6.4</td>
<td>Hormonal stimulation</td>
<td>21</td>
</tr>
<tr>
<td>2.6.5</td>
<td>Contamination of milt</td>
<td>22</td>
</tr>
<tr>
<td>2.7</td>
<td>Short term preservation of milt</td>
<td>22</td>
</tr>
<tr>
<td>3.</td>
<td><strong>MATERIALS AND METHODS</strong></td>
<td>25-29</td>
</tr>
<tr>
<td>3.1</td>
<td>Selection of brooders and maintenance</td>
<td>25</td>
</tr>
<tr>
<td>3.2</td>
<td>Hormonal inducement</td>
<td>26</td>
</tr>
<tr>
<td>3.3</td>
<td>Collection and processing of milt</td>
<td>27</td>
</tr>
<tr>
<td>3.4</td>
<td>Observation of Physical and Chemical properties of milt</td>
<td>27</td>
</tr>
<tr>
<td>3.5</td>
<td>Observations of spermatological parameters</td>
<td>27</td>
</tr>
<tr>
<td>3.5.1</td>
<td>Sperm density</td>
<td>28</td>
</tr>
<tr>
<td>3.5.2</td>
<td>Motility test</td>
<td>28</td>
</tr>
<tr>
<td>3.5.3</td>
<td>Percentage of live and dead sperm</td>
<td>29</td>
</tr>
<tr>
<td>3.6</td>
<td>Short-term preservation</td>
<td>29</td>
</tr>
<tr>
<td>3.7</td>
<td>Statistical analysis</td>
<td>29</td>
</tr>
<tr>
<td>4.</td>
<td><strong>RESULT</strong></td>
<td>30-45</td>
</tr>
<tr>
<td>4.1</td>
<td>Physical properties of milt</td>
<td>30</td>
</tr>
</tbody>
</table>
4.2 Sperm density 32
4.3 Spermatological parameters after inducement with Ovatide 32
4.4 Spermatological parameters after inducement with WovaFH 34
4.5 Spermatological parameters after inducement with Ovaprim 38
4.6 Statistical analysis 38
4.7 Comparative analysis of the influence of hormones and their doses on the motility duration of spermatozoa 44

5. DISCUSSION 46-52
5.1 Sperm density 46
5.2 Percentage of motile cells 46
5.3 Motility duration 46
5.4 Short term preservation 49

6. SUMMARY 53-56

7. REFERENCES 57-97
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Title</th>
<th>Page no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Range of water quality parameters observed and recorded in the brooder tank</td>
<td>24</td>
</tr>
<tr>
<td>2.</td>
<td>Details of hormone treatment</td>
<td>25</td>
</tr>
<tr>
<td>3.</td>
<td>Motility score prescribed by Betsy and Stephen (2014)</td>
<td>28</td>
</tr>
<tr>
<td>4.</td>
<td>Physical properties of the milt collected from <em>C. carpio</em> after hormonal inducement</td>
<td>30</td>
</tr>
<tr>
<td>5.</td>
<td>Initial mean values of spermatological parameters of milt collected from male <em>C. carpio</em> subjected to different hormonal inducements immediately after collection</td>
<td>33</td>
</tr>
<tr>
<td>6.</td>
<td>Mean motility duration (s) of <em>C. carpio</em> spermatozoa induced with Ovatide at two different doses and observed at 24 h interval during short term storage</td>
<td>35</td>
</tr>
<tr>
<td>7.</td>
<td>Mean motility score of spermatozoa of <em>C. carpio</em> induced with Ovatide at two different doses and observed at 24 h interval during short term storage</td>
<td>35</td>
</tr>
<tr>
<td>8.</td>
<td>Mean motility pattern of spermatozoa of <em>C. carpio</em> induced with Ovatide at two different doses and observed at 24 h interval during short term storage</td>
<td>35</td>
</tr>
<tr>
<td>9.</td>
<td>Mean motility duration (s) of spermatozoa of <em>C. carpio</em> induced with Wova-FH at two different doses and observed at 24 h interval during short term storage</td>
<td>37</td>
</tr>
<tr>
<td>Sl. No.</td>
<td>Title</td>
<td>Page no.</td>
</tr>
<tr>
<td>--------</td>
<td>----------------------------------------------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>10.</td>
<td>Mean motility score of spermatozoa of <em>C. carpio</em> induced with Wova-FH</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>at two different doses and observed at 24 h interval during short term storage</td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>Mean motility pattern of spermatozoa <em>C. carpio</em> induced with Wova-FH</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>at two different doses and observed at 24 h interval during short term storage</td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td>Mean motility duration (s) given to spermatozoa of <em>C. carpio</em> induced with Ovaprim at two different doses and observed at 24 h interval during short term storage</td>
<td>39</td>
</tr>
<tr>
<td>13.</td>
<td>Mean motility score of spermatozoa of <em>C. carpio</em> induced with Ovaprim</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>at two different doses and observed at 24 h interval during short term storage</td>
<td></td>
</tr>
<tr>
<td>14.</td>
<td>Mean motility pattern of spermatozoa <em>C. carpio</em> spermatozoa induced with Ovaprim at two different doses and observed at 24 h interval during short term storage</td>
<td>40</td>
</tr>
<tr>
<td>15.</td>
<td>Estimated changes in motility duration</td>
<td>40</td>
</tr>
<tr>
<td>16.</td>
<td>Statistical analysis of the results obtained for <em>C. carpio</em> spermatozoa collected after inducement and short term preserved</td>
<td>40</td>
</tr>
</tbody>
</table>
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Title</th>
<th>Page no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Mean spermatozoa density in milt collected from <em>C. carpio</em> after and before inducement with three different hormones at two different doses</td>
<td>33</td>
</tr>
<tr>
<td>2.</td>
<td>Rate of change in motility duration of <em>C. carpio</em> spermatozoa induced with Ovatide at two different concentrations</td>
<td>36</td>
</tr>
<tr>
<td>3.</td>
<td>Rate of change in motility duration of short term preserved <em>C. carpio</em> spermatozoa induced with wova-FH at two different concentrations</td>
<td>36</td>
</tr>
<tr>
<td>4.</td>
<td>Rate of change in motility duration of short term preserved <em>C. carpio</em> spermatozoa induced with Ovaprim at two different concentrations</td>
<td>36</td>
</tr>
<tr>
<td>5.</td>
<td>Percentage of live and dead spermatozoa of <em>C. carpio</em> milt induced with Ovatide at two different doses and observed on 120h of short term preservation</td>
<td>41</td>
</tr>
<tr>
<td>6.</td>
<td>Percentage of live and dead spermatozoa of <em>C. carpio</em> milt induced with Wova-FH at two different doses and observed on 120h of short term preservation</td>
<td>42</td>
</tr>
<tr>
<td>7.</td>
<td>Percentage of live and dead spermatozoa of <em>C. carpio</em> milt induced with Ovaprim at two different doses and observed on 120h of short term preservation</td>
<td>42</td>
</tr>
<tr>
<td>8.</td>
<td>Percentage reduction in the motility performance of <em>C. carpio</em> spermatozoa collected after inducement with Ovatide at two different levels</td>
<td>43</td>
</tr>
<tr>
<td>9.</td>
<td>Percentage reduction in the motility performance of <em>C. carpio</em> spermatozoa</td>
<td>43</td>
</tr>
<tr>
<td>Sl.No.</td>
<td>Title</td>
<td>Page no.</td>
</tr>
<tr>
<td>-------</td>
<td>----------------------------------------------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td></td>
<td>collected after inducement with Wova-FH at two different levels</td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td>Percentage reduction in the motility performance of <em>C. carpio</em> spermatozoa collected after inducement with Ovaprim at two different levels</td>
<td>43</td>
</tr>
</tbody>
</table>
## LIST OF PLATES

<table>
<thead>
<tr>
<th>Title</th>
<th>Between pages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plate-1</strong></td>
<td></td>
</tr>
<tr>
<td>• Experimental fish, <em>Cyprinus carpio</em></td>
<td>37</td>
</tr>
<tr>
<td><strong>Plate-2</strong></td>
<td></td>
</tr>
<tr>
<td>• Hapa installed in cement pond at National Fish Seed Farm, Manimuthar where the breeders were maintained</td>
<td>39</td>
</tr>
<tr>
<td>• Male brooder of <em>C. carpio</em> being weighed for hormone dose calculation</td>
<td>39</td>
</tr>
<tr>
<td>• Male <em>C. carpio</em> given with hormone injection</td>
<td>40</td>
</tr>
<tr>
<td><strong>Plate-3</strong></td>
<td></td>
</tr>
<tr>
<td>• Collection of milt at Manimuthar Dam</td>
<td>41</td>
</tr>
<tr>
<td>• Collection of milt from the hormonally induced male <em>C. carpio</em> during the first trial</td>
<td>41</td>
</tr>
<tr>
<td>• Collection of milt from the hormonally induced male <em>C. carpio</em> during the second trial</td>
<td>42</td>
</tr>
<tr>
<td><strong>Plate-4</strong></td>
<td></td>
</tr>
<tr>
<td>• Milt being analysed in the laboratory</td>
<td>46</td>
</tr>
<tr>
<td>• Diluted milt observed under microscope (200X)</td>
<td>46</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

World aquaculture production during the year 2012 was 66.6 million tonnes which has evidenced an annual growth rate of 6.1% (FAO, 2014). In this, world food fish aquaculture production was 44.2 million tonnes. India occupied second place with the total aquaculture production of 4.2 million tonnes (FAO, 2014). During the year 2011, per capita fish protein supply was 1.6 g per day in India. Thus, fish contributed 2.6% to the total protein supply of the country (FAO, 2014). Common carp is the widely cultured fish throughout the world and it has been cultured in 98 countries and China was the principal producer of common carp with the production of 2.89 million tonnes (FAO, 2014). The total production of common carp during the year 2012 was 3.79 million tonnes with the value of 5,207,971,000 USD (FAO, 2014).

Common carp (*Cyprinus carpio* Linneaus, 1758) has been cultivated for several thousands of years. It is an omnivore and feeding on everything available in the water. It is native to Europe. It breeds in ponds. The original Prussian strain of common carp was introduced in India by the Britishers during 1939 in the Otkamund Lake. It grows faster than Rohu or Mrigal. Common carp attains sexual maturity much earlier than the Indian Major Carps and it grows even after maturity. The age at first maturity is 6-8 months and the males mature earlier to the female. In temperate countries, common carp is a seasonal breeder whereas, in tropical countries like India, it is a perennial breeder and matured fishes are available throughout the year. Common carp is a prolific breeder capable of spawning several times in a year (Badapanda, 2012).
There are three varieties of Common carp, *viz.*, *C. carpio* var. *communis*, *C. carpio* var. *specularis*, and *C. carpio* var. *nudus*. The body of *C. carpio* var. *communis* is fully covered by regularly arranged rows of scales, and this is the original form of common carp with normal colouration. *C. carpio* var. *specularis* is called as Mirror Carp and its body is covered unevenly with a few large and bright scales. *C. carpio* var. *nudus* is known as Leather Carp and its body is almost devoid of scales except for a single row of some degenerated scales along the base of the dorsal fin and sometimes extending as far as the tail or even from head to tail (Saad and Billard, 1987).

Carps are generally living in lotic environment and they can do well in large lentic waters. They breed in flowing waters like river and they never breed in small confined waters. The seed collected from natural resources is generally a mixed stock with both desired and undesired varieties.

Overcoming problems related to seed quality, induced breeding is considered advantageous to get pure and required quantity of fish seed. Through induced breeding, pure seeds of desirable species can be obtained (Murthy *et al.*, 2013).

Common carp do breed in captivity. The reason may be either environmental or hormonal. Certain environmental parameters like photoperiods, rain, temperature, water current, etc. can influence the hormonal activity from pituitary and gonads. Disturbances arise in environment may cause insufficient release of hormones in captive conditions and thus, sometimes they do not breed in captivity.

The technique of induced breeding was first evolved in Argentina using pituitary extract by Houssay in 1930. Brazil was the first country to induce fish by injection of pituitary gland.
During 1934, Von-Ihering of Brazil succeeded in breeding Brazilian fishes by administration of fish pituitary hormone (Von-Ihering, 1935). In 1938, Russian scientist, Gerbilskii succeeded in breeding sturgeon using pituitary hormones. USA was the third country to induce fish by neurohormonal treatment. Hasler has bred Pike by hypophysation as early as 1939 and the technique was mainly employed for breeding Minnow. In 1970, east European countries induced Chinese grass carp to breed successfully. In China, during 1964, Chinese carps were successfully bred by hypophysation technique using fish pituitary gland.

In India, Khan in 1938 was the first to induce Mrigal (Cirrhinus mrigala), by injecting anterior lobe of the mammalian pituitary extract. Following him, Ramaswamy and Sunderaraj in 1956 induced Singhi (Heteropneustes fossilis) and Minnow (Esomus danricus). Chaudhury and Alikunhi (1957) successfully induced Rohu (Labeo rohita), Mrigal (C. mrigala), Reba (C. reba), and Bata (Labeo bata), at CMFRI, Orissa. After three years, in 1960, all the species of Indian Major Carps were bred by hypophysation in the same station.

In induced breeding technique, two types of hormones are used, namely natural hormone and synthetic hormone. Salmon Gonadotropin Releasing Hormone (sGnRH) or Luteinising Hormone Releasing Hormone (LHRH) analogues were found to be effective in fish breeding (Lin and Peter, 1996). Pituitary gland extraction is a well-established technique for induced breeding all over the world and Human Chorionic Gonadotropin (HCG) has been found as an alternative for pituitary gland. There are problems in using these hormones, such as weighing, low quantity, preparation of these analogues and storage of these prepared solutions. Hence to overcome these problems, synthetic hormones are used. Synthetic hormones contain GnRH and dopamine blocker receptor. Ovaprim, Ovatide, Wova- FH, Dagin and Aquaspawn are
commercially very popular and found to be efficient in successful spawning of fishes (Peter et al., 1988; Nandeesha et al., 1990).

Ovaprim was introduced in the market as a substitute for pituitary gland extract. It is a synthetic hormone manufactured by Syndel Laboratories Inc, Vancouver, British Columbia, Canada. All the fish were found to respond positively for this drug (Nandeesha et al., 1990). Ovaprim contains analogue of salmon GnRH and dopamine inhibitor (Domperidone) required for cultivable fishes. Another important synthetic hormone was Ovatide which was launched by Hemmapharma, Mumbai. It is also a combination of GnRH analogue with dopamine antagonist, Pimozide. Wova-FH can be used for induced breeding in carp and catfish. It is a synthetic Gonadotropin analogue; dosage of Wova-FH can be adjusted depending on the brood stock, weather and agro climate conditions (Aknakali et al., 2011).

Even though hormonal inducement can make the fish to breed, the sperm quality plays an important role in determining the fertilization success. Spermatology is the study about various spermatological parameters like sperm motility, sperm density, pH, motility score and motility pattern. The motility is the most commonly used parameter to evaluate sperm quality in fishes (Billard et al., 1995, Lahnsteiner et al., 1998). Studies involving fish spermatozoa were done as early as 1853 (Quatrefages, 1853). The study included fishes like pike, carp, perch, loach and barbs and this research has been said to be beneficial for the improvement of seed production.

In commercial fish farming, the evaluation of sperm qualities is important to increase the efficiency of hatchery seed production. High individual variations of sperm quality are frequently reported (Rana, 1995). This may be due to sex ratio, stocking density, age, size, nutrition and feeding regime (Ridha and Cruz, 1989).
Fish farming industry has been more concerned on the quality of eggs or larvae rather than that of sperm, even though the quality of both gametes may affect fertilization success and larval survival (Rurangwa et al., 2004). In teleost species, evaluation of male gametes for their quality has been primarily dependent on sperm motility, sperm concentration and spermatocrit (Suquet et al., 1992; Rana, 1995).

Improved technique for storage and evaluation of fish sperm would enhance breeding programs around the world (Segovia et al., 2000). Artificial insemination requires a large quantity of good quality semen. Collection and storage of good quality semen can improve artificial insemination by reducing the stress to male brood stock caused by repeated semen sampling that reduces semen quality (Ritar, 1999). Sperm can be stored and refrigerated for periods ranging from a few hours to several weeks, depending on the species (Scott and Baynes, 1980). The preservation of fish sperm for short term duration is generally useful from the commercial point of view and facilitates various hatchery operation. The short term storage of sperm at low temperature (4°C) is mostly applied. The fish sperm could be preserved by storage in undiluted and diluted form (Sahin et al., 2013). The semen of Salmonids has been successfully stored for 14-15 days (Buyukhatipoglu and Holtz, 1978; Stoss and Refstie, 1983). Semen of rainbow trout retained full fertilizing capacity for up to seven days (Babiak and Dabrowsky, 2003).

Considering the importance of studying the sperm quality of *C. carpio* when induced with different hormones and its changes during short term preservation, the present research was carried out with the following objectives.
Objectives

• To study the spermatological parameters of the milt from *C. carpio* obtained after inducement with different hormones at varying doses.

• To compare the spermatological parameters of milt from induced and naturally maturing *C. carpio* adults of the same population.

• To quantify the variations in the spermatological parameters of *C. carpio* due to hormonal inducement.

• To determine the suitable hormone and doses for the production of milt with optimum speramntological quality.
2. REVIEW OF LITERATURE

2.1. Hormones

Hormone is a chemical substance released by specific endocrine gland that can serve as a messenger possessing specific or diverse actions with some other organs (Fsgbenro et al., 1991). Hormone action is particularly related to metabolic activities that are anabolic and catabolic in the cytoplasm. The nervous and endocrine mechanism use basic properties of living cells, such as secretion and the propagation of impulses (Fsgbenro et al., 1991). The hypothalamus in vertebrates contains certain neurosecretary cells specialized for the production of hormones. In many cases neurons secrete a hormone for synaptic transmission (Fsgbenro et al., 1991). Endogenous control is mediated through actions of various hormones along the brain-hypothalamus-pituitary-gonad axis (Chaudhuri and Alikunhi, 1957). In fish, gametogenesis is controlled by the reproductive hormones of the brain, pituitary and gonad (Chaudhuri, 1955). Under natural conditions, environmental stimuli are detected and relayed to the brain, resulting in release of hormones and neurotransmitters that regulate ovulation (Yaron, 1995; Peter and Yu, 1997).

The endocrine control of reproduction in finfish is based on the hypothalamus-hypophysis (pituitary gland)- gonad axis. Hypothalamus produces the factor Gonadotropin-releasing hormone whenever there are environmental stimuli which act on the pituitary gland. Pituitary gland controls synthesis and release of gonadotropin hormones (GtHs), which stimulate the gonads to produce gametes (Rainis et al., 2003). In animal, Gonadotropin-releasing hormone (GnRH) initiates animal endocrine pathway stimulating the reproductive events in a more balanced and integrated way, with other physiological functions related to the reproduction cycle (Mylonas et al., 1966). Therefore, it can be said that GnRH also stimulates the release of other
pituitary hormones involved in the reproductive cycle, like growth hormone (GH), thyroid stimulating hormone (TSH) and somatolactine (Mylonas et al., 1998; Zohar and Mylonas, 2001). Nerve cells which cytologically show signs of a secretory activity are found in many animals. In vertebrates, they were described originally by Dahlgren (1914) and Speidel (1919; 1922) in the hindmost part of the spinal cord of rays and teleosts. The discovery of gland like cells in the spinal cord of sharks by Dahlgren (1914) was the earliest observation of the secretory activity of nerve cells. This observation was later confirmed by Speidal (1919) in skates. Dahlgren studied electric organs and reported an association between the special neurons and the presence of electric organs. Further, he described certain glandular cells from the preoptic nucleus (PON) of the hypothalamus of the minnow (Phoxinus laevis) and hinted on their endocrine function. Ernst and Seafarer (1934) have added an impressive body of evidence for the existence of neurosecretory cells in the nervous system of invertebrates and vertebrates (Scharrer and Scabarrer, 1954). According to Rath (2011) during 1940s, neurosecretory cells were found ending up in the neurohypophysis (nervous party of hypophysis) and by neural mechanism, monitored hormonal secretions of the hypophysis.

2.2. Induced breeding of fishes

A common problem in Cyprinid aquaculture is obtaining good quality seeds for stocking (Kulikovsky et al., 1996; Horvath et al., 1997). Adequate and reliable supply of fingerlings is a prerequisite in aquaculture. Collection of fingerlings from the wild is not reliable because of seasonal availability and less quantity. Many fishes do not breed in captivity, the reason for which may be environmental and consequently hormonal. Disturbances arising in environment may influence release of meager amount of hormone in captive conditions. The environmental parameters like photoperiod, rain, temperature, water current, etc. influence the hormonal
activity from pituitary and gonads. Hence induced breeding technique was developed to offer a reliable solution to overcome the problem of non-availability of fingerlings in aquaculture.

Induced Breeding is a method in which pituitary extract or any other hormone preparation is injected into the body of matured parent fish for maturity induction and spawning (Heggberget, 1996). Although natural spawning is the preferred method of breeding of cultivated fish, induced spawning is felt useful in timing and synchrony of egg production (Marte, 1989). The failure of the reproduction can be prevented by induced breeding attempts as expressed by Kulkarni and Ogale (1986).

Aschheim and Zondek (1927) were the first person to do experiment on the role of pituitary in the reproduction of invertebrates. The concept of pituitary application for successful spawning in fish was first developed by Houssay (1930) of Argentina. Brazil was the first country to develop a technique for hypophysation by Von Ihering (1935), Von Ihering and his co-workers, Martins, Cardoso, de Azevedo and others conducted experiments with various hormone injections on the lines of Houssay (1930) and achieved success in 1934 (Rath, 2011). Since then, Brazilian fish culturists have been employing this technique to obtain seeds from indigenous fishes as a part of their routine piscicultural programmes.

Impressed by the work of Brazilians, the Russians introduced hormone treatment for fishes in their country (Von Ihering, 1935). Following the success reported by Gerbilskii (1938), this method was used in the Soviet Union for production of sturgeon eggs in the farms situated along the lower Volga, Ural, Kuban and other rivers (Gerbilskii, 1965). Since then several workers have used this procedure for breeding in different parts of the world.

The first hypophysial attempt in China was adopted in 1958 by aquaculture researchers of Guangdong province by injecting the extracts of hypophysis of common carp into brood fish of
Silver Carp and Bighead Carp cultured in ponds. In the same year, researchers of Zhejiang province also succeeded in induced spawning of silver carp and bighead carp by applying human chorionic gonadotropin (hCG). In 1960, artificial propagation of Grass Carp in China was successfully done by using pituitary gland of common carp (Nandeesh et al., 1990).

In India, the first induced breeding attempt was made in 1937 (Khan, 1938), using mammalian pituitary extract. Chaudhry (1955) successfully induced Indian Major Carps using fish pituitary gland extract. He also bred *Pseudotropius atherinoides* (Bloch) by administering pituitary extract from *Cirrhinus reba*. Chaudhry and Alikunhi (1957) succeeded in inducing IMCs and minor carps using carp pituitary extract. In 1962, induced breeding of Chinese carps was successfully done by employing similar technique (Alikunhi *et al*., 1963). Chondar (1970) and Varghese *et al*. (1975) stated that with the help of induced breeding technique, Indian major carps and Chinese carps can be made to breed three times in the same season, thereby the quantity of seeds produced could be enhanced.

### 2.3. Inducement with natural hormone

Maturation of spermatozoa in fish takes place in the spermatic ducts and is hormonally controlled (11-ketotestosterone and 17α, 20β-dihydroxy-4-pregn-3-one), while the entire process of spermatogenesis is controlled and influenced by environmental factors and linked to the species reproduction strategy (Yaron, 1995). In fish, milt production can be stimulated by the treatment with either GtH or GnRH and their synthetic analogues (GnRHa) (Zohar and Myloans, 2001).
It has been found that inducing reproduction with hormonal substances can affect the volume of milt and the motility of spermatozoa (Redono-Muller et al., 1991). Injections of commercially available hormonal substances like natural gonadotropins *i.e.*, Human Chorionic Gonadotropin (HCG), Carp Pituitary Homogenate (CPH), or Carp Pituitary Extract (CPE) or Bream Pituitary Homogenate (BPH). Natural Gonadotropin-Releasing Hormone (GnRH) and analogues (sGnRH, mGnRHa), have proven effective when applied in aquaculture to induce ovulation and stimulate spermiation in fish (Brzuska and Adamec, 1999; Szabo et al., 2002; Szabo, 2003; Kucharczyk et al., 2008; Targonska et al., 2010). Volume of milt and quantity of spermatozoa in cyprind fishes are influenced by many factors including the time gap for spawning (Billard, 1986; Saad and Billard, 1987; Caille et al., 2006; Cejko et al., 2010).

### 2.3.1. Pituitary extracts


### 2.3.2 Human Chorionic Gonadotropin
Human Chorionic Gonadotropin (HCG) is a Luteinizing Hormone (LH)-like substance that can be used successfully for the induced breeding of fish (Ohta and Tanaka, 1997). It has been used to stimulate fish reproduction similar to synthetic GnRHa (Kucharczyk, 2002). It stimulated the spermiation process by acting on gonadal maturation (Miura et al., 1991). It has been reported that single administration or weekly injections of HCG induced spermatogenesis in Japanese and European eel (Khan et al., 1987; Miura et al., 1991; Ohta, 1996; Ohta and Tanaka, 1997; Perez et al., 2000). A single hCG injection can induce 13 fold increase in stripped sperm volume in Pangasius becourti (Cacot et al., 2003).

Among the mammalian gonadotropins, Human Chorionic Gonadotropin (HCG) is effective in inducing spermatogenesis and spermiation in fish (Stacey and Peter, 1979; Donaldson and Hunter, 1983). In fresh water Salmon (Oncorhynchus keta), it has been reported that HCG stimulated advancement of spermiation (Crim et al., 1982; Sorenson and Pankhursts, 1988) and promoted an increase in milt volume (Takashima et al., 1984; Kreiberg et al., 1987; Yueh et al., 1990).

In Japanese eel, HCG treatment led to production of a small volume (Ohta and Tanaka 1997), while injections of Pituitary Extracts in adult carp (Cyprinus carpio) and rainbow trout (Oncorhynchus mykiss) decreased sperm concentration, by increasing seminal fluid production (Clemens and Grant, 1965). This increased seminal fluid normally lead to the decreased density of the spermatozoa as observed by Dabrowski et al. (1994).

2.4. Inducement with synthetic hormones

2.4.1. Ovatide

“Ovatide” is structurally related to the naturally occurring, Goanadotropin Releasing Hormone (GnRH) which is a peptide with low viscosity (Kahkesh et al., 2010). The dosage for
males ranged from 0.10-0.20 ml/kg for Rohu, Mrigal and Calbasu, 0.20-0.30 ml/kg. Catla required for 0.20-0.25 ml/kg and Silver Carp and Grass Carp also.

Marimuthu et al. (2009) reported that Ovatide gave high fertilization and hatching percentage (85-90%) in carps. It increased egg production through complete spawning and produced healthy seeds. Due to its low viscosity, it was easy to inject and it did not cause any adverse effect in brood fish after injection. Similar to Ovaprim, it can be administered in a single dose to brooders and it can be stored at room temperature. Ovatide has been used to induce breeding in different fish species in India. The following table required dose for species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Dosage</th>
<th>Hormone</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Catla catla</em></td>
<td>0.2-0.5 ml/kg</td>
<td>Ovatide</td>
<td>Thakur and Reddy 1997</td>
</tr>
<tr>
<td><em>L. calbasu</em></td>
<td>0.3-0.5 ml/kg</td>
<td>Ovatide</td>
<td>Thakur and Reddy 1997</td>
</tr>
<tr>
<td><em>Labeo rohita</em></td>
<td>0.2-0.4 ml/kg</td>
<td>Ovatide</td>
<td>Thakur and Reddy 1997</td>
</tr>
<tr>
<td><em>Cirrhinus mrigala</em></td>
<td>0.2-0.4 ml/kg</td>
<td>Ovatide</td>
<td>Thakur and Reddy 1997</td>
</tr>
<tr>
<td><em>Ctenopharyngodon idella</em></td>
<td>0.2-0.65 ml/kg</td>
<td>Ovatide</td>
<td>Thakur and Reddy 1997</td>
</tr>
<tr>
<td><em>Puntius javanicus</em></td>
<td>0.3-0.6 ml/kg</td>
<td>Ovatide</td>
<td>Thakur and Reddy 1997</td>
</tr>
</tbody>
</table>

*Heteropneustes fossilis* and *Channa strriatus* is 0.4 ml/kg (Marimuthu et al., 2007). Reported optimum dose of ovatide for *Channa punctatus* was 0.2-0.6 ml/kg (Marimuthu et al., 2009).

2.4.2. Wova-FH

Wova-FH (WOCKHARDT) has been used for induced breeding in carp and catfish (Akankali et al., 2011). The trade name is WOVA-FHTM-Synthetic Gonadotropin analogue (WoVA-FHTM-SnGnRHa). Performance of WOVA-FHTM has been assessed in some of the
government and private hatcheries in Andra Pradesh, West Bengal, Orissa and other places (Motilan et al., 2014).

<table>
<thead>
<tr>
<th>Species</th>
<th>Sex</th>
<th>Dosage</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catla, Rohu and Mrigal</td>
<td>male</td>
<td>0.1-0.30 ml/kg BW</td>
<td>Akankali et al. (2011)</td>
</tr>
<tr>
<td></td>
<td>female</td>
<td>0.3-0.50 ml/kg BW</td>
<td>Akankali et al. (2011)</td>
</tr>
<tr>
<td>Silver carp, Grass carp and Cat fish</td>
<td>male</td>
<td>0.1-0.40 ml/kg BW</td>
<td>Akankali et al. (2011)</td>
</tr>
<tr>
<td></td>
<td>female</td>
<td>0.4-0.80 ml/kg BW</td>
<td>Akankali et al. (2011)</td>
</tr>
</tbody>
</table>

2.4.3. Ovaprim

“Ovaprim” is a complex, synthetic substance containing Salmon hypothalamus hormone analogue (D-Arg6, Pro9Net-SGnRh) and domperidone (a dopamine receptor antagonist) (King and Young, 2001; Zohar and Mylonas, 2001; Hanifa et al., 2007). The use of sGnRHa resulted in successful stimulation of ovulation in Cyprinds (Drori et al., 1994; Hill et al., 2005).

There are many advantages with the use of Ovaprim. It is a ready to use product and the solution is stable at ambient temperature (Seifi et al., 2011). The ovaprim contains an analogue of 20µg of salmon Gonadotropin Releasing Hormone (sGnRHα) and 10µg domperidone, It has been adjudged as the most convenient and effective ovulating agent (Piska and Naik, 2007). Ovaprim has been reported to have unique advantages over pituitary hormone.

Although the effective dose required for various species of carp is found to vary considerably (Rurangwa et al., 2004), Ovaprim gave encouraging results in Catla, Rohu, Mrigal, silver carp, grass carp and big head carp with inducement of maturation (Seifi et al., 2011). Male common carp is 0.10-0.20 ml ovaprim /kg body weight, while exotic carp (silver carp, grass grill) were bred at doses ranging from 0.40-0.60 ml/kg body weight. Big head carp was
bred successfully at a dose of 0.5 ml/kg body weight. The method of injection is the same as that of pituitary (Piska and Naik, 2007).

According to Nandeesha et al. (1990), the required dose of Ovaprim for mrigal was 0.3 ml/kg. While Exotic carps required 0.15-0.20 ml/kg body weight (Piska and Naik, 2007); Caspin brown trout, *salmo truttacaspius* required 0.5 ml/kg body weight (Farahi et al., 2012); *Aspius aspius* required 0.25 ml/kg body weight (Ceiko et al., 2008). Based on the observations of Nandeesha et al. (1990 and 1993), the dosage of Ovaprim required for female brood fish of various species are *Catla catla* required 0.40 to 0.50 ml/kg, *Labeo rohita* required 0.30 to 0.40 ml/kg, *Cirrhinus mrigala* required 0.25 to 0.30 ml/kg, *Hypophthalmichthys molitrix* required 0.50 to 0.70 ml/kg, *Ctenopharyngodon idella* required 0.50-0.70 ml/kg, Big head carp required 0.50 ml/kg, *Labeo bata* required 0.50 ml/kg, and Fringe-lipped carp required 0.50 ml/kg. In ovaprim recommended dose for *Catla catla, Labeo rohita, Cirrhinus mrigala* required 0.4-0.6 ml/kg (More et al., 2010). Saud et al. (2013) reported the dose for *Labeo rohita* required 0.2-0.4 ml/kg body weight.

2.5. Spermatological parameters of fish spermatozoa

The quality of sperm from individual male fishes not only depends on the genome, but also on many other factors (Suquet et al., 2000). The sperm quality can be determined by analyzing several parameters like motility, spermatocrit, fertilizing capacity, osmolality, pH of seminal plasma and chemical composition of seminal plasma (Rurangwa et al., 2004).

2.5.1. Colour and Viscosity

There can be slight variation in the colour of milt among different species but there will be great variation in the viscosity of the milt. Kruger (1984) visually compared the colour and viscosity of *Cyprinus carpio* milt immediately after stripping. He observed that the colour was
creamy white in both common carp and Tilapia. Linhart et al. (1986) stated that the sperm of Tench (*Tinca tinca*) and Wels (*Silurus glanis*) was milky white colour.

Volk and Van vuren (1988) used a 3 scale point (1-3) to evaluate viscosity in the milt of small mouth yellow fish (*Mycteroperca interstitialis*). The semen of Atlantic Blue Fin Tuna *Thunnus thynnus* was highly viscous, like a white oil paint (Doi *et al.*, 1982). Therefore it is understood that uniformity in colour can not be expected among different species for their milt.

### 2.5.2. Sperm Count:

The sperm density can be determined accurately with a haemocytometer (Kolmer and Boerner, 1941). Tvedt *et al.* (2001) also used haemocytometer counting chamber to determine the spermatozoa density. Truscott and Idler (1969) reported that the concentration of sperm cells in semen of Atlantic Salmon ranged from $12 \times 10^9$ to $30 \times 10^9$ cells /ml. Billard *et al.* (1971) recorded the sperm concentration of Rainbow Trout and said that it was ranging from $6 \times 10^9$ to $25 \times 10^9$ cells/ml of ejaculate. Billard *et al.* (1983) and Takashima *et al.* (1984) estimated the number of spermatozoa by densitometric method.

Clemens and Grant (1965) reported that the sperm cells densities were inversely proportional to the water content of semen and testes in common carp. Sanchez-Rodriquez *et al.* (1978) showed that the spermatocrit packed cell volume or total semen volume remained constant throughout the spawning period.

### 2.5.3. Motility duration

The spermatozoa motility indicates the semen quality and it is the criterion commonly used in the selection of milt for insemination and preservation. One of the important parameters in fish sperm quality evaluation is the analysis of motility (Billard *et al.*, 1995; Lahnsteiner and
Fish spermatozoa are immotile in the testis (Stoss, 1983). It was first shown by Scheuring (1924); Gaschott (1924) and later by Schlenk and Kahmann (1938) that the potassium in the seminal plasma prevented motility in fish sperm. The spermatozoa motility varies in vigor and duration not only among males but also within an individual male depending on its ripeness. In numerous fish species with external fertilization, duration of spermatozoa motility has been reported to be very short ranging from few seconds to few minutes (Billard and Cosson, 1989).

The percentage of motile spermatozoa was defined as the percentage of spermatozoa actively swimming in forward motion. Takai and Morisawa (2011) judged only forward moving sperm as motile and sperm cells that vibrated in place were considered to be immotile.

The motility duration is often evaluated only after diluting the milt with suitable diluent. Billard and Cosson (1992) pre-diluted the semen in a saline solution at a ratio of 1/100 and observed the motility duration. Another dilution in a physiological serum at a ratio of 1/20 was made and 1µl of solution was placed on the microscope stage and observed for fixing motility duration by a semi-quantitative method by Rurangwa et al. (2004).

The sperm motility was evaluated using a light microscope (X400) and was expressed in seconds and the motility also expressed as percentage of motile spermatozoa (Bozkurt et al., 2005). The motility durations were evaluated by the mass progressive motility when most of the spermatozoa were still actively swimming with progressive movement, total duration of movement until 90% of the spermatozoa stopped swimming (Sahin et al., 2013).

The motility might be related to the particular environmental conditions including temperature, salinity, pH, season and fish species during the spawning (Lahnsteiner et al., 1995).

**2.5.4. Milt pH**
The pH of seminal plasma has a greater role in determining the motility initiation and its duration (Seifi et al., 2011). The fluctuations in semen pH of salmonid fish are related to the secretion of bicarbonate into seminal plasma by the spermatic duct epithelium during final maturation of the spermatozoa (Morisawa and Morisawa, 1988). The low semen pH in brood stock may be related to a deficiency in bicarbonate secretion by the spermatic duct epithelium during the early spawning season (Lahnsteiner et al., 1996; Billard et al., 1989). Farahi et al. (2012) reported that the semen pH and osmolality in brood stock of Caspian Brown Trout after 3 days of hormonal stimulation was lower than that of other groups.

Several studies showed significant correlations between the mineral content of seminal plasma and osmolality (Alavi and Cosson, 2006). Thus, the differences in semen osmolality can depend on changes in the mineral content and some organic compounds of the seminal plasma at different times of spermiation during the spawning period (Hajirezaei et al., 2010).

### 2.6 Factors affecting milt quality

Factors like season, temperature, nutrition, stress, hormonal stimulation, milt contamination and short-term storage are known to affect the quality of spermatozoa (Ciereszko, 2008). It has been found that inducing reproduction with hormonal substances can affect the volume of milt and the motility of spermatozoa (Redondo-Muller et al., 1991). Injections of commercially available hormonal substances like natural gonadotropins *i.e.*, Human Chorionic Gonadotropin (HCG), Carp Pituitary Homogenate (CPH), or Carp Pituitary Extract (CPE) or Bream Pituitary Homogenate (BPH), as well as natural Gonadotropin Releasing Hormone (GnRH) and analogues (sGnRH, mGnRHa) have proven effective when applied in aquaculture to
induce ovulation and stimulate spermiation in fishes (Brzuska and Adamek 1999; Szabo et al., 2002; Szabo, 2003; Kucharczyk et al., 2008; Targonska et al., 2010).

Volume of milt and quantity of spermatozoa in Cyprinid fishes are influenced by many factors including the time gap for spawning (Billard, 1986; Saad and Billard, 1987; Caille et al., 2006; Cejko et al., 2010). Volume of milt that could be obtained by regular stripping has been measured for Salmo ischcahan (Turdakov, 1968) and for S. gairdneri (Billard, 1977; Sanchez-Rodriquez et al., 1978). The volume of milt was increased with increasing age (Khodzher, 1981; Khorevin, 1987), and length and weight of the fish (Khorevin, 1987). The relative volume of sperm obtained from single stripping was higher in lower weight trout (Linhart, 1984).

There are many factors influencing the sperm density of matured males. Jaspers (1972) noted that age seemed to be one of the major factors influencing sperm concentration as 3 years old fish producing more spermatozoa per gram wet testicular tissue than 2 years old. The concentration of spermatozoa found to decrease with age, length, and weight of fish (Khorevin, 1987). Hormonal stimulation resulted in slight decrease in sperm count/ml of milt but the total number of sperms collected after hormone injection was higher due to the increase in volume (Saad and Billard, 1987). Farahi et al. (2012) stated that the time after hormonal stimulation did not affect spermatozoa motility and sperm density.

2.6.1 Seasonal changes

According to Ciereszko et al. (1996) and Ciereszko (2004) fish seminal plasma has a significant influence due to season. Christ et al. (1996) reported that common carp sperm production and quality can be lower at the beginning and end of the breeding season. Similar variations based on season could be noticed in other carps also.

2.6.2 Temperature
Reproductive development in temperate fish is regulated by photoperiod and water temperature. Photothermal manipulations often result in significant changes in semen quality, including sperm concentration and sperm motility characteristics in common carps (Dabrowski et al., 1996; Ciereszko et al., 1998). Temperature affects the semen characteristics of fishes (Williot et al., 2000). Emri et al. (1998) found higher sodium concentrations, pH values, and lower potassium concentrations in seminal plasma of cold-adopted common carp as compared to warm-adopted carp.

2.6.3 Age of broodstock

Age of the broodstock has significant influence on the sperm quality and it affects the success of storing of sperm (Vuthiphandchai and Zohar, 1999). Based on higher sperm production and increased sperm longevity during short term storage, captive reared three years Old Striped Bass (Morone saxatilis) had better sperm quality than 1-12 year old fish (Vuthiphandchai and Zohar, 1999).

2.6.4 Hormonal stimulation

Milt of captive fish is low in volume and high in sperm concentration. It also appears very viscous. The results of hormonal stimulation are increase in the milt volume and decrease in sperm concentration. An increase in the volume is attributed to increased milt availability and fluidity (Clemens and Grant, 1965; Vermeiressen et al., 2000; Moon et al., 2003). According to Vermeiressen et al. (2004), the possible reasons for milt hydration are enhanced secretion and reduced resorption of water.

Clearwater and Crim (1998) reported that increase in seminal plasma pH has also been reported in GnRHa stimulated Yellowtail Flounder (Pleuronectes ferrugineus). This increase
was accompanied by better sperm motility characteristics like percentage of motile cells and duration of motility. Moon et al. (2003) reported a positive correlation between seminal plasma pH and sperm motility for milt of GnRHa stimulated Starry Flounder (Platichthys stellatus).

Hormonal induction is known to increase the fluidity of the milt and low concentration of spermatozoa was found in Plaice, Pleuronectes platessa (Vermeirssen et al., 1998), winter flounder P. americanus (Shangguan and Crim, 1999) and Atlantic Halibut H. hippoglossus (Vermeirssen et al., 2000). In common carp, oral and intraperitoneal administration of salmon Gonadotropin Releasing hormone analogue (sGnRHa) and Pimozide induced gonadotropin Hormone II (GtH II) caused milt production significantly (Roelants et al., 2000).

2.6.5 Contamination of milt

Sperm quality and seminal plasma composition are greatly affected by milt contamination (Glogowski et al., 2000). Milt collected by stripping method may lead to contamination of milt with mucus, feaces, blood, water or urine (Ciereszko et al., 2004). Contamination of milt with blood led to changes in composition of seminal plasma, especially the protein profile due to the higher protein concentration in blood (Loir et al., 1990; Ciereszko et al., 1998; Rinchard et al., 2003). A potential milt contaminant is faecal matter due to the introduction of bacteria. During short term storage of milt, microbial growth is an inevitable problem. Hence faeces contaminated milt is not suitable for storage. Satterfield and Flickinger (1995) recorded reduction in storage time of Walleye (Stizostedion vitreum) milt from 11.7 days in Control semen to 2 days in milt contaminated with faecal matter.

In some farmed fish species like European cat fish, Silurus glanis (Linhart and Billard, 1994), common carp, C. carpio (Billard et al., 1995; Perchec et al., 1995; 1998), Turbot, Psetta maxima (Dreanno et al., 1999), Tilapia, Oreochromis mossambicus, tench, Tinca tinca (Linhart
and Kvasnicka, 1992), urine initiated spontaneous sperm movement was noticed. Contaminated carp milt had decreased endogenous storage of energy and motility (Perchec et al., 1995; 1998) which is a characteristic of premature induction of motility.

2.7. Short term preservation

Short term preservation is one of the oldest methods for storing milt for a short period for use. Experiments were conducted involving the storage of salmonid sperm at temperature from 0-9º C (Scheuring, 1924; Smith and Quistorff, 1943; Withler and Morley, 1962; Stein, 1975). Improved techniques for storage and evaluation of fish sperm would enhance breeding programs around the world (Segovia et al., 2000).

The short term storage technique was found to be a useful tool for the genetic development of aquaculture species as it could help in the transfer of genes from wild stock to hatchery stock and maintain the genetic diversity. Short-term storage of sperm can be used to synchronize male and female gamete availability, aid in the transport of semen and reduce the risk of disease transmission.

Sperm can be refrigerated and stored for periods ranging from a few hours to several weeks, depending on the species (Scott and Baynes 1980). Semen from common carp has been successfully stored at 0° to 5° C for up to 5 h (Hulata and Rothbard, 1979), while that for Rainbow Trout it was stored at -2° C for up to three weeks (Stoss et al., 1978).

Short term preservation is done on for fishes like, Salmonids (Scott and Baynes, 1980; Billard, 1981), Milk fish (Chanos chanos) (Hara et al., 1982; Erdhal et al., 1984), Tilapia (Tilapia mossambicus) (Erdhal and Graham, 1987 and McNiven et al., 1993), Walleye (Stizostedion vitreum) (Moore, 1987; Jensen and Alderdice, 1982), Atlantic Sturgeon (Acipenser oxyrinchus) (Dilauro et al., 1994), Paddle fish (Polyodon spathula) (Brown and Mims, 1995), Striped Bass
Atlantic salmon (*Salmo salar*) sperm when stored at 5-6° C, the spermatozoa maintained high level of fertility up to 2.5 days (Brofelt, 1923). Henderson and Dewar (1959) reported that short term preservation of undiluted sperm of brook Trout (*Salvelinus fontinalis*), helped to achieve higher hatching yield in 5 days under the chilled condition. Truscott et al. (1968) stated that the milt of same species was stored at 2-3° C and the fertilization was possible. Dushkin (1975) reported that when sperm of Pacific Herring (*Clupea pallasi*) was stored at 0.8 to 1° C, fertility can be retained for 3 weeks. Stoss et al. (1987) observed better survival rates of Rainbow Trout sperm until day 16 when kept undisturbed.
3. Materials and Methods

3.1. Selection of brooders and maintenance

Male *Cyprinus carpio* with mean body weight of 600 ± 70g were selected (Plate1 and 2b) from the culture pond and separately maintained for milt collection as described by Billard *et al.* (1983). Brooders were stocked at a density of 1kg per m$^3$ and fed twice a day with brooder diet containing Ground Nut Oil Cake, Rice Bran and Wheat Bran (Gissi *et al.*, 1991). Water quality parameters such as Temperature, dissolved oxygen, pH, ammonia and nitrate were estimated once in a week in the brooder pond during day time (Lahnsteiner *et al.*, 1995; Lin and Peter, 1996; Azuadi *et al.*, 2013) and the observations recorded are given in Table 1. Fishes with rough pectoral fin back were considered matured and selected for inducement (Jhingran and Pullin, 1985;)

Table 1. Range of water quality parameters observed and recorded in the broodertank

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Parameters</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Temperature ($^{0}$ C)</td>
<td>27- 30$^{0}$ C</td>
</tr>
<tr>
<td>2</td>
<td>Dissolved Oxygen (mg/l)</td>
<td>2.8-5.4</td>
</tr>
<tr>
<td>3</td>
<td>pH</td>
<td>7.2-8.0</td>
</tr>
<tr>
<td>4</td>
<td>Ammonia</td>
<td>0.01 to 0.07 ppm</td>
</tr>
<tr>
<td>5</td>
<td>Nitrate</td>
<td>0.01 to 0.03 ppm</td>
</tr>
<tr>
<td>6</td>
<td>Depth of water</td>
<td>1.0 to 1.25 m</td>
</tr>
</tbody>
</table>
3.2 Hormonal Inducement

Three hormones *viz*., Ovatide, Wova-FH and Ovaprim were selected for the hormonal inducement. In the preliminary study, these hormones were administered to males at the recommended rate of 0.5 ml/kg of body weight (Farahi *et al.*, 2012), in order to find out the experimental range of hormones to be administered to determine their influence on the spermatozoa quality. Hormones were injected intramuscularly (Plate-2c) (Thomas *et al.*, 2003) at the base of the caudal region during early hours of the day (Ahmadnezhad *et al.*, 2012). The injected male carps were maintained separately in hapas for 5 hours (Marte, 1989) before collection of milt (Plate-2a). This time interval was found to be sufficient to ensure complete spermiation as recommended by Billard *et al.* (1995). The collected milt was observed for
different parameters. After the observation of the preliminary results, the hormones were fixed at two different doses viz., 0.3 and 0.6 ml/kg body weight. The details of the treatments designed for the present study are given in Table 2.

Table 2. Details of hormone treatments in the present study

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Treatment</th>
<th>Hormone</th>
<th>Doses (ml/ kg body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ot₁</td>
<td>Ovatide</td>
<td>0.3</td>
</tr>
<tr>
<td>2</td>
<td>Ot₂</td>
<td>Ovatide</td>
<td>0.6</td>
</tr>
<tr>
<td>3</td>
<td>Wf₁</td>
<td>Wova-FH</td>
<td>0.3</td>
</tr>
<tr>
<td>4</td>
<td>Wf₂</td>
<td>Wova-FH</td>
<td>0.6</td>
</tr>
<tr>
<td>5</td>
<td>Op₁</td>
<td>Ovaprim</td>
<td>0.3</td>
</tr>
<tr>
<td>6</td>
<td>Op₂</td>
<td>Ovaprim</td>
<td>0.6</td>
</tr>
<tr>
<td>7</td>
<td>C</td>
<td>Control</td>
<td>0</td>
</tr>
</tbody>
</table>

(Ovatide- Ot, Wova-FH- Wf, Ovaprim- Op)

3.3 Collection and processing of milt

Milt was collected by stripping method as suggested Billard et al. (1995). Before collection, the genital pore region was cleaned with absorbent cotton and double ply tissue paper to remove the moisture and mucus. The urine and faecal matter were removed by gentle press near the genital pore and care was taken to avoid blood contamination (Lubzens et al., 1997). Gentle pressure was given in the ventral portion nearer to the pelvic fins and the milt oozed out from the genital pore was collected carefully in a pre- labelled, marked sterile cryovial (Plate-3 a,b,c) (Ghaffer et al., 2004). The collected milt was carefully sealed and kept in the ice box initially with gel ice to bring them down to 16-20°C (Akankali et al., 2011). The samples were
then taken for processing in the cryopreservation lab after 2 hours of collection.

3.4 Observation of physical properties of milt

Physical properties of milt like colour, volume of milt and milt pH were assessed following the methods suggested by Kruger et al. (1984). Milt pH was evaluated by placing a drop of milt in pH indicator strips (pH: 0-14; Merck, Germany) (Billard et al., 1989). The volume of milt was determined from the graduations in the cryovials. The colour of the milt was ascertained by visual observation (Rurangwa et al., 2004).
PLATE -3

a

b

c
3.5 Observation of spermatological parameters

Spermatological parameters such as sperm density, motility duration, percentage of motile cells and motility pattern were observed in the diluted samples. The observations were made using NIKON E360 microscope under phase contrast at 200× magnification.

3.5.1 Sperm density

The sperm density was determined using the standard haemocytometer (NAUBAEUR, Germany). The mean number of cells per square (n) was arrived at after counting the spermatozoa present in approximately 10 squares from different locations. The multiplication factor was calculated based on the number of squares taken for counting sperm cells, depth and area of the observation pane in haemocytometer and dilution rate of the milt sample. The cell concentration was estimated using the formula,
\[ C = \frac{n \times N \times DF}{v} \]

where,

- \( C \) = Cell concentration /ml
- \( n \) = Average number of spermatozoa per square
- \( N \) = Total number of squares in the haemocytometer
- \( v \) = Volume of sample
- \( DF \) = Dilution Factor

### 3.5.2 Motility test

Motility duration was estimated by placing 10µl of milt on the grease free glass slide, to which 10µl of water was added (Plate-4b). The motility duration was estimated from the time the spermatozoa exhibited movement till 50% of the sample lost its movement (Seifi et al., 2011). Motility score was assigned to each sample following Betsy and Stephen (2014) as (Table 3). The motility pattern was determined as described by Nomura (1964).

#### Table 3. Motility score prescribed by Betsy and Stephen (2014)

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Motility score</th>
</tr>
</thead>
<tbody>
<tr>
<td>All spermatozoa (95-99%) progressively motile with various flagella movements.</td>
<td>10</td>
</tr>
<tr>
<td>Most spermatozoa (90-95%) progressively motile, while others exhibit strong vibration with forward movement.</td>
<td>9</td>
</tr>
<tr>
<td>Most spermatozoa (85-90%) progressively motile, while others exhibit weak</td>
<td>8</td>
</tr>
</tbody>
</table>
vibration with forward movement.

<table>
<thead>
<tr>
<th>Percentage</th>
<th>Description</th>
</tr>
</thead>
</table>
| 7          | Most spermatozoa (80-85%) exhibit strong vibration with forward movement, while others vibrate in *loco*.
| 6          | Most spermatozoa (75-80%) exhibit weak vibration with forward movement, while others vibrate in *loco*.
| 5          | All spermatozoa (90-95%) exhibit strong vibration in *loco*.
| 4          | All spermatozoa (90-95%) exhibit weak vibration in *loco*.
| 3          | Most spermatozoa (85-90%) exhibit strong vibration in *loco* while others oscillate.
| 2          | Most spermatozoa (85-90%) exhibit weak vibration in *loco* while others oscillate.
| 1          | Most spermatozoa (75-85%) oscillate while others vibrate.
| 0.75       | Most spermatozoa (60-75%) vibrate while others are immotile.
| 0.5        | All spermatozoa (90-95%) oscillate.
| 0.25       | Most spermatozoa oscillate while others are immotile.
| 0          | All spermatozoa immotile.

### 3.5.3 Percentage of live and dead spermatozoa

The percentage of live and dead spermatozoa was assessed using Eosin-Nigrosin stain as described by Chutia *et al.* (1998). On grease free glass slides 1µl of diluted milt was mixed with 1µl Eosin-Nigrosin stain placed on the corner of the slide. With the help of cover glass, the milt and stain were dragged front and back for proper mixing. The smears were dried at 40° C in hot plate for 10 to 20 s. The percentage of live and dead spermatozoa was calculated by the concept
of stain exclusion by the living cells. This was ascertained by microscopic observation (Martoja and Martoja, 1967).

**PLATE-4**

a

![Image of a person working with microscopes]

b
3.6 Short term preservation

The diluted fish milt was observed for the spermatological qualities during for a short term preservation of 3-4 days at low temperature of 0-4°C as suggested by Rana (1995). The collected milt was processed using 0.85% physiological saline solution and the milt was diluted 40 times with the diluents in a beaker (Plate-4a). The beaker was covered with aluminium foil to prevent air contamination and the covered beaker was placed in the refrigerator at 4°C for 5 days. Observation was made once in 24 h on all the spermatological parameters for 120 hours. Before observation, the diluted milt was brought to room temperature.
3.7. Statistical analysis

All the observed data were processed and the results were statistically analysed with ANOVA and paired 't' test for finding at the significance.
4. RESULTS

Observations on various physical and spermatological parameters of the milt collected after inducement with three different hormones at two different concentrations and stored for short term at refrigerated condition are given below in Tables 4 to 16 and depicted in Figures 1 to 10.

4.1. Physical properties of milt

The volume of milt collected from fishes induced with different hormones at two different doses ranged from 0.8 to 1.0 ml. The mean volume of milt collected from the males in individual treatment is given in Table 4. The milt was milky white in colour and the pH of the milt ranged from 7.1 to 7.4.

Table 4. Physical properties of the milt collected from *C. carpio* after hormonal inducement

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Ovatide (ml/kg)</th>
<th>Wova-FH (ml/kg)</th>
<th>Ovaprim (ml/kg)</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.3</td>
<td>0.6</td>
<td>0.3</td>
<td>0.6</td>
</tr>
<tr>
<td>Mean Volume (ml)</td>
<td>0.8±0</td>
<td>1.0±0</td>
<td>0.9±0</td>
<td>1.0±0</td>
</tr>
<tr>
<td>Colour</td>
<td>Milky white</td>
<td>Milky white</td>
<td>Milky white</td>
<td>Milky white</td>
</tr>
<tr>
<td>Mean pH</td>
<td>7.1±0</td>
<td>7.2±0</td>
<td>7.3±0</td>
<td>7.2±0</td>
</tr>
</tbody>
</table>
4.2 Sperm density

The densities observed for the milt collected from the fishes induced with Ovatide, Wova-FH and Ovaprim at doses of 0.3 and 0.6 ml/kg of BW are given in Table 5. There were variations in the sperm density for different hormone doses. The average sperm density was $3.2 \pm 0.01 \times 10^9$ cells/ml. The sperm density differed with different hormones and different doses.

The sperm density decreased with increased dose of hormone. The highest sperm density of $4.3 \times 10^9$ cells/ml was observed when Ovaprim was used at a dose of 0.3 ml/kg of BW followed by Wova-FH ($4.1 \times 10^9$ cells/ml) and Ovatide ($3.8 \times 10^9$ cells/ml) at 0.3 ml/kg of BW. In contrary to this, when hormones were given at a dose of 0.6 ml/kg BW, the sperm density was found to be the lowest ($1.2 \times 10^9$ cells/ml); it was better for Ovatide ($1.8 \times 10^9$ cells/ml) and for Wova-FH it was still better ($2.8 \times 10^9$ cells/ml) as depicted in Fig. 1.

The initial mean values for various spermatological parameters such as motility duration, motility percentage and motility score observed for the milt collected from different treatments are given in Table 5.

The mean motility duration of spermatozoa collected from naturally matured fish (Control) was $134 \pm 20.8$ s. This value was higher than the motility duration of spermatozoa collected from fishes induced with Ovatide, Wova-FH and Ovaprim at 0.3 ml/kg of BW, but the motility duration of spermatozoa from Control group was lower than that of fishes treated with hormones at 0.6 ml/kg BW. The initial motility score of milt collected from induced and naturally matured males was 10 whereas the motility percentage ranged between 97 and 99%.

Changes that took place in the spermatological parameters of *C. carpio* collected after different hormone treatments were presented in Tables 6-14. There was significant variation
among the treatments for the density of spermatozoa for different doses of hormones administrated (P>0.01) (Table 5). On 120 h of observation, the motility duration of Control decreased to 33 ± 70 s (Tables 6, 9, 12). There were significant differences in the motility duration between Control and Op2 and Control and Wf2 (Table 16). The values were statistically significant (P>0.01) when analysed with Paired ‘t’ test.

Similar results were observed for motility score (Tables 7, 10, 13) and motility pattern (Tables 8, 11, 14). Initially the motility score for all the samples was 10. The reduction in the score was slow till 72 h in all the treatments. In Control, the motility score value decreased drastically to 3 from 8 on 96 h of observation. On 120 h, the value came down to 0.5. There was forward movement initially, that changed to circular movement after which it changed its pattern to vibrating movement. The percentage of live cell was 99% on first day which came down to 39% on 5th day of observation.

4.3 Spermatological parameters after inducement with Ovatide

The spermatozoa collected from the male C. carpio induced with Ovatide at 0.3 ml/kg BW had the initial mean motility duration of 123 ± 16.0 s. It decreased to 93 ± 6.12 s when observed after 24 h. After 120 h, the mean motility duration came down to 41 ± 4.08 s as seen in Table 6. Similar reduction in the mean motility score was also observed (Table 7). The initial mean motility score was 10, which decreased to 6 on 48 h for Ot1. On 5th day, the mean motility score was 0.5 (Table 7). On first day alone, the spermatozoa exhibited forward movement after which it showed circular movement until 72 h. It showed vibrating movement at the end of 120 h of observation (Table 8). Initially the percentage of live cells was 99% which decreased to 38% on 5th day as shown in Fig. 5.
Table 5. Initial mean values of spermatological parameters of milt collected from male *C. carpio* subjected to different hormonal inducements immediately after collection

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Ovatide (ml/kg BW)</th>
<th>Wova-FH (ml/kg BW)</th>
<th>Ovaprim (ml/kg BW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.3</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>Sperm density (×10^9 cells/ml)</td>
<td>3.5±0.02</td>
<td>3.8±0.05</td>
<td>1.8±0.1*</td>
<td>4.1±0.3</td>
</tr>
<tr>
<td>Motility duration (s)</td>
<td>134±20.8</td>
<td>123±16</td>
<td>178±1.75</td>
<td>116±10.5</td>
</tr>
<tr>
<td>Motility percentage</td>
<td>99</td>
<td>98</td>
<td>99</td>
<td>97</td>
</tr>
<tr>
<td>Motility score</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

(* P>0.01)

Fig. 1. Mean spermatozoa density in milt collected from *C. carpio* matured naturally and induced with three different hormones at two different doses
The initial mean motility duration of spermatozoa collected from males injected with Ovatide at a dose of 0.6 ml/kg BW was 178±1.75 s. After 24 h, the mean motility duration decreased to 150 ± 3.0 s. The value decreased drastically to 60±4.28 s on 120 h of observation (Table 6). There was significant difference between the spermatozoa from Ot1 and Ot2 groups (P>0.01). The rate of change in motility duration is depicted in Fig. 2. It can be understood that the initial mean motility score of 10 was reduced to 0.75 on 5th day (Table 7). The spermatozoa exhibited forward movement on 1st day and changed its pattern to vibrating movement on 5th day (Table 8). On 5th day, the percentage of live cells was 40% (Fig. 5).

4.4 Spermatological parameters after inducement with Wova-FH

The initial mean motility duration of spermatozoa collected from C.carpio induced with Wova-FH at 0.3 ml/kg BW was 116 ± 10.5 s. The value decreased to 78 ±2.86 s at 48 h and to 29 ± 6.5 s at 120 h of observation (Table 9). The rate of change in the motility duration was depicted in Fig. 3. When observed the mean motility score was 10 when observed at 1st day. On 2nd, 3rd and 4th day, the mean motility score reduced to 9, 7, and 6, respectively (Table 10). Similar variations were observed in the motility pattern also (Table 11). On first day, the percentage of live cells were 98% which reduced down to 36% on 120 h (Fig. 6).

When Wova-FH was given at 0.6 ml/kg BW, the initial mean motility duration was 176±1.75 s. It decreased steadily and reached 51±4.5 s on 120 h, which is higher than that of the Control (33±70 s) (Table 9). The values were statistically significant between Wf1 and Wf2 and between C and Wf2 (P>0.01). The initial motility score in all the treatments was 10. The value decreased to 0.75 on 5th day in milt collected from fish induced with 0.6 ml/kg BW. Similar
changes were observed in the motility score and motility pattern (Table 10 and 11). The percentage of live cells was 39% on 5th day of observation as it can be seen in Fig. 6.

Table 6. Mean motility duration (s) of C. carpio spermatozoa induced with Ovatide at two different doses and observed at 24 h interval during short term storage

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hours</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>96</th>
<th>120</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ot₁</td>
<td>Initial</td>
<td>123±16.0</td>
<td>93±6.12</td>
<td>74±8.5</td>
<td>61±8.17</td>
<td>50±7.5</td>
<td>41±4.08</td>
</tr>
<tr>
<td>Ot₂</td>
<td>Initial</td>
<td>178±1.75</td>
<td>150±3.0</td>
<td>108±2.91</td>
<td>92±5.0</td>
<td>75±3.5</td>
<td>60±4.28</td>
</tr>
<tr>
<td>Control</td>
<td>Initial</td>
<td>134±20.8</td>
<td>117±7.24</td>
<td>84±13.9</td>
<td>63±12.43</td>
<td>55±6.94</td>
<td>33±70</td>
</tr>
</tbody>
</table>

*P>0.01

Table 7. Mean motility score given to spermatozoa of C. carpio induced with Ovatide at two different doses and observed at 24 h interval during short term storage

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Hours</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>96</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ot₁</td>
<td>Initial</td>
<td>10</td>
<td>9</td>
<td>6</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Ot₂</td>
<td>Initial</td>
<td>10</td>
<td>10</td>
<td>9</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Control</td>
<td>Initial</td>
<td>10</td>
<td>9</td>
<td>8</td>
<td>8</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 8. Mean motility pattern of spermatozoa of C. carpio induced with Ovatide at two different doses and observed at 24 h interval during short term storage

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Hours</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>96</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ot₁</td>
<td>Initial</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Ot₂</td>
<td>Initial</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Control</td>
<td>Initial</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>
Fig. 2. Rate of change in motility duration of short term preserved \textit{C. carpio} spermatozoa induced with Ovatide at two different concentrations

Fig. 3. Rate of change in motility duration of short term preserved \textit{C. carpio} spermatozoa induced with Wova-FH at two different concentrations

Fig. 4. Rate of change in motility duration of short term preserved \textit{C. carpio} spermatozoa induced with Ovaprim at two different concentrations
Table 9. Mean motility duration (s) of spermatozoa of *C. carpio* induced with Wova-FH at two different doses and observed at 24 h interval during short term storage

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Hours</th>
<th>Initial</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>96</th>
<th>120</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wf&lt;sub&gt;1&lt;/sub&gt;</td>
<td></td>
<td>116±10.5</td>
<td>98±3.03</td>
<td>78±2.86</td>
<td>53±8.15</td>
<td>43±2.44</td>
<td>29±6.5</td>
<td>70±30.71</td>
</tr>
<tr>
<td>Wf&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td>176±1.49</td>
<td>158±5.90</td>
<td>105±2.0</td>
<td>87±1.63</td>
<td>68±6.5</td>
<td>51±4.5</td>
<td>108±45.5*</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>134±20.8</td>
<td>117±7.24</td>
<td>84±13.9</td>
<td>63±12.43</td>
<td>55±6.94</td>
<td>33±70</td>
<td>81±35.16</td>
</tr>
</tbody>
</table>

*P>0.01

Table 10. Mean motility score of spermatozoa *C. carpio* induced with Wova-FH at two different doses and observed at 24 h interval during short term storage

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Hours</th>
<th>Initial</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>96</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wf&lt;sub&gt;1&lt;/sub&gt;</td>
<td></td>
<td>10</td>
<td>9</td>
<td>7</td>
<td>6</td>
<td>3</td>
<td>0.50</td>
</tr>
<tr>
<td>Wf&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td>10</td>
<td>10</td>
<td>9</td>
<td>8</td>
<td>5</td>
<td>0.75</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>10</td>
<td>9</td>
<td>8</td>
<td>8</td>
<td>3</td>
<td>0.50</td>
</tr>
</tbody>
</table>

Table 11. Mean motility pattern of spermatozoa *C. carpio* induced with Wova-FH at two different doses and observed at 24 h interval during short term storage

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Hours</th>
<th>Initial</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>96</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wf&lt;sub&gt;1&lt;/sub&gt;</td>
<td></td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Wf&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td>+++</td>
<td>+++</td>
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<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
4.5 Spermatological parameters after inducement with Ovaprim

The initial mean motility duration of spermatozoa collected from fish induced with 0.3 ml/kg BW Ovaprim was 110±6.23 s. The motility duration decreased to 86±2.86 s in 48 h and further to 27±1.69 s in 120 h as it can be seen in Table 12. Similar reduction in the mean motility score was also observed. The initial mean motility score was 10, which decreased to 7 on 48 h. On 5th day, the mean motility score was 0.5 (Table 13). The spermatozoa exhibited forward movement on first day which changed to circular movement on 72 h. It showed vibrating movement during 120 h of observation (Table 14). Initially the percentage of live cells was 98%. On 5th day of observation, the percentage of live cells came down to 37% as shown in Fig.7.

When Ovaprim was injected at 0.6 ml/kg BW, the initial mean motility duration was 153 ± 6.12 s. The mean motility duration was 125 ± 4.18 s, 112 ± 5.90 s, 100 ±1.24 s when observed at 24, 48 and 72 h, respectively (Table 12). The rate of change in motility duration is depicted in Fig. 4. The values were statistically significant between Op₁ and Op₂ and between C and Op₂ (P>0.01). The statistical analysis of the results obtained are presented in Table 16. The initial mean motility score was 10 which reduced to 8 on 72 h as seen in Table 13. The spermatozoa exhibited forward movement on 1st, 2nd and 3rd day after which the pattern changed to vibrating movement on 5th day (Table 14). The percentage of live cells was reduced to 38% on 120 h of observation as given in Fig. 7.

4.6. Statistical analysis

Statistical analysis of the data observed in the present study revealed that the treatments such as Op₂ (Ovaprim at 0.6 ml/kg BW) and Wf₂ (Wova-FH at 0.6 ml/kg BW) were statistically significant from the Control with respect to the motility duration. Other treatments such as Op₁, Ot₁, Ot₂ and Wf₁ are not significantly different from the Control. However, there was significant
difference among their doses such as 0.3 ml/kg BW and 0.6 ml/kg BW. Ot₁, Ot₂ and Wf₁ are not significantly different from the Control. However, there was significant difference among their doses such as 0.3 ml/kg BW and 0.6 ml/kg BW.

Table 12. Mean motility duration (sec) given to spermatozoa of *C. carpio* induced with Ovaprim at two different doses and observed at 24 h interval during short term storage

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Hours</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>96</th>
<th>120</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Op₁</td>
<td>Initial</td>
<td>10</td>
<td>9</td>
<td>7</td>
<td>6</td>
<td>3</td>
<td>0.50</td>
</tr>
<tr>
<td>Op₂</td>
<td>Initial</td>
<td>10</td>
<td>10</td>
<td>9</td>
<td>8</td>
<td>3</td>
<td>0.75</td>
</tr>
<tr>
<td>Control</td>
<td>Initial</td>
<td>10</td>
<td>9</td>
<td>8</td>
<td>8</td>
<td>3</td>
<td>0.50</td>
</tr>
</tbody>
</table>

*P>0.01

Table 13. Mean motility score of spermatozoa of *C. carpio* induced with Ovaprim at two different doses and observed at 24 h interval during short term storage
Table 14. Mean motility pattern of spermatozoa *C. carpio* spermatozoa induced with Ovaprim at two different doses and observed at 24 h interval during short term storage

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Initial</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>96</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Op1</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Op2</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Control</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 15. Estimated changes in motility duration

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Treatments</th>
<th>Overall mean % reduction</th>
<th>$R^2$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>C</td>
<td>35.6±16.13</td>
<td>0.9793</td>
</tr>
<tr>
<td>2.</td>
<td>Ot1</td>
<td>19.4±2.4</td>
<td>0.5087</td>
</tr>
<tr>
<td>3.</td>
<td>Ot2</td>
<td>19.0±4.97</td>
<td>0.1972*</td>
</tr>
<tr>
<td>4.</td>
<td>Wf1</td>
<td>23.4±7.2</td>
<td>0.6541</td>
</tr>
<tr>
<td>5.</td>
<td>Wf2</td>
<td>21.2±6.61</td>
<td>0.6796</td>
</tr>
<tr>
<td>6.</td>
<td>Op1</td>
<td>26.6±9.3</td>
<td>0.7713</td>
</tr>
<tr>
<td>7.</td>
<td>Op2</td>
<td>22.8±7.08</td>
<td>0.786</td>
</tr>
</tbody>
</table>

(*$P>0.01$)
Table 16. Statistical analysis of the results obtained for *C. carpio* spermatozoa collected after inducement and short term preserved

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Calculated t value</th>
<th>Table t value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Op₁ Vs Op₂</td>
<td>5.89</td>
<td>4.03</td>
<td>0.01</td>
</tr>
<tr>
<td>Ot₁ Vs Ot₂</td>
<td>5.67</td>
<td>4.03</td>
<td>0.01</td>
</tr>
<tr>
<td>C Vs Op₂</td>
<td>15.28</td>
<td>4.03</td>
<td>0.01</td>
</tr>
<tr>
<td>Wf₁ Vs Wf₂</td>
<td>5.96</td>
<td>4.03</td>
<td>0.01</td>
</tr>
<tr>
<td>C Vs Wf₂</td>
<td>24.9</td>
<td>4.03</td>
<td>0.01</td>
</tr>
</tbody>
</table>
Fig. 5. Percentage of live and dead spermatozoa of *C. carpio* milt induced with Ovatide at two different doses and observed on 120 h of short term preservation.

Fig. 6. Percentage of live and dead spermatozoa of *C. carpio* milt induced with Wova-FH at two different doses and observed on 120 h of short term preservation.
Fig. 7. Percentage of live and dead spermatozoa of *C. carpio* milt induced with Ovaprim at two different doses and observed on 120 h of short term preservation.

Fig. 8. Percentage reduction in the motility performance of *C. carpio* spermatozoa collected after inducement with Ovatide at two different levels.
Fig. 9. Percentage reduction in the motility performance of *C. carpio* spermatozoa collected after inducement with Wova-FH at two different levels

![Graph showing data for Wova-FH](image)

R² = 0.7713  
R² = 0.786  
R² = 0.9793

Fig. 10. Percentage reduction in the motility performance of *C. carpio* spermatozoa collected after inducement with Ovaprim at two different levels

![Graph showing data for Ovaprim](image)

R² = 0.6541  
R² = 0.6796  
R² = 0.9793
4.7. Comparative analysis of the influence of hormones and their doses on the motility duration of spermatozoa

Comparative analysis of the data pertaining to motility duration of spermatozoa of *C. carpio* induced with hormones at different doses is presented in Table 15 and Fig 8 to 10. The overall mean motility reduction percentage of spermatozoa collected from normally matured fish (Control) was 35.6±16.13. The value was higher when compared to other treatments. On first day of observation, the reduction in motility percentage was found to be 12%. The reduction increased steadily and reached 42% and 61% during 96 h and 120 h of observation, respectively (Fig. 8).

Overall mean motility reduction percentage of spermatozoa collected from Ovatide induced males (0.3 ml/kg BW) was 19.4±2.4 (Table 15). On 24 h, The percentage of motility reduction was 24% after which the reduction percentage decreased steadily and reached 18% at the end of 120 h. When Ovatide was given at 0.6 ml/kg BW, the motility reduction percentage was 19.0±4.97. The motility reduction percentage during 24 h was 15% which was very low compared to the reduction percentage when Ovatide was given at 0.3 ml/kg BW as given in Figure 8.

The spermatozoa collected from fishes induced with Wova-FH at 0.3 ml/kg BW had a motility reduction percentage of 23.4 ± 7.2 (Table 15). The motility reduction percentage during 24 h was 15% which increased to 32% during 72 h and 120 h. The mean motility reduction percentage was lower (21.2±6.61) when Wova-FH was given at 0.6 ml/kg BW. Initially, the reduction percentage was slower (10%) at 24 h which reached its peak (30%) on 72 h. Then the reduction percentage came down to 21% and 25% on 96 h and 120 h, respectively which can be seen in the Fig. 9.
When Ovaprim was given at 0.3 ml/kg BW, the mean motility reduction percentage was 26.6±9.3. This value was higher when compared to that of all the other treatments except Control. On 24 h, Ovaprim 0.3 ml/kg BW the motility reduction percentage was 23% which decreased to 19% on 48 h respectively. At the end of 120 h, the motility reduction percentage was 44%. Mean motility reduction percentage of spermatozoa collected from Ovaprim induced males at 0.6 ml/kg BW was 22.8±7.08. The motility reduction percentage on 24 h was 11%, which rose to 20% on 72 h. During 120 h of observation, the motility reduction percentage was 27% (Fig. 10).
5. DISCUSSION

5.1 Sperm Density

Density of the spermatozoa in fish milt has been reported to be without any consistant figures. The densities of spermatozoa of *Cyprinus carpio* induced with three different hormones at two different doses were found to be within a range of $1.2 \times 10^9$ to $4.3 \times 10^9$ cells per ml. Sperm density is an essential factor in the determination of sperm quality (Suquet *et al*., 1992). Babiak *et al*. (1997) found that the sperm density was $20 \times 10^9$ cells per ml of milt in *C. carpio*. In *C. carpio var communis*, Chutia *et al*. (1998) estimated the sperm density as $6.6 \times 10^9$ cells per ml of milt. Lahnsteiner *et al*. (2000) reported that sperm density of *C. carpio* was 0.5 to $1.0 \times 10^{11}$ cells per ml of milt. The sperm density of Mirror carp was found to be $17.33 \pm 1.22 \times 10^9$ cells/ml as reported by Akcay *et al*. (2004).

In the present study, the mean sperm density was found to be $3.2 \pm 0.5 \times 10^9$ cells/ml. The highest mean sperm density of $4.3 \pm 0.25 \times 10^9$ cells/ml was found when Ovaprim was given at 0.3 ml/kg BW and the lowest sperm density was found with *C. carpio* when injected with Ovaprim at 0.6 ml/kg BW ($1.2 \pm 0.1 \times 10^9$ cells/ml). It is also worth mentioning that the sperm density was inversely proportional to the concentration of hormone. As observed in Table 5, all the hormones at 0.3 ml/kg BW dose resulted in higher densities than that of 0.6 ml/kg BW hormone dose. The values obtained for sperm density although were not in accordance with the reports by various authors (Saad *et al*., 1988; Babiak *et al*., 1997; Chutia *et al*., 1998 and Akcay *et al*., 2002), it is to be noted that the sperm density is always inconsistent as noted by Emri *et al*. (1998). The difference in the sperm density in the present study may be due to hormonal inducement,
differences in feeding conditions, age, environmental factors, time of spawning, or dilution ratio as reported by Emri et al. (1998).

5.2 Percentage of motile cells

Next to density of spermatozoa, the presence of some viable cells in the milt is the next important factor deciding the quality of milt. In the milt collected from fish which was naturally matured, the percentage of motile cells was 99%. When Ovatide was given at 0.3 and 0.6 ml/kg BW, the percentage of motile cells was 99% whereas, Wova-FH and Ovaprim at 0.3 and 0.6 ml/kg BW showed 98% motile cells initially. This was similar to the results of Redono-Muller et al. (1991) who reported that hormonal injection did not change the initial percentage of motile sperm and it was 100% in common carp semen from hormonally treated fish.

Therefore, it can be said that the increased hormone doses when not altering the volume of milt, will have lower spermatozoa densities due to higher volume of seminal plasma, as observed and reported by Akcay et al. (2002 and 2004).

5.3 Motility Duration

The motility of spermatozoa is considered as a reliable index of semen quality (Terner, 1986). Motility features of the spermatozoa are reported to be depending on many factors such as feeding conditions, age, environmental factors, time of spawning or dilution ratio (Bozkurt, 2005). This is found applicable in fishes too. Saad et al. (1988) stated that the spermatozoa of most freshwater fishes have a limited duration of motility. Benau and Terner (1980), Kruger et al. (1984) and Akcay et al. (2004) reported that the motility duration can vary seasonally. According to Kruger et al. (1984) average time of carp spermatozoa motility ranged from 1.2 to 1.6 minutes based on the seasons. Spermatozoa motility varies in vigor and duration not only
among males but also in an individual male depending on the stage of maturity (Akcay et al., 2002). Physiological studies of the males and their maturation process are reported to influence the motility features of the spermatozoa in fishes (Zohar and Mylonas, 2001).

Jezierska (1994) and Jezierska et al. (1995) although reported that the maximum motility time of carp sperm was 60 s, there were some more authors who reported more motility time than 60 s, for carp (Stoss, 1983; Alavi and Cosson, 2005, 2006). Falling in line with the observation above mentioned, in the present study also, initial mean motility duration in Control was 134±20.8s. Dilution in seminal plasma or complex saline solutions that mimic the composition of the seminal plasma did not significantly improve sperm survival as observed and reported by Mims (1991).

The initial mean motility duration was 178±1.75 s when Ovatide was given at 0.6 ml/kg BW. This duration is 44.72% higher than that of the samples collected after inducement with Ovatide at 0.3 ml/kg BW. It can be also said that all the hormones at 0.6 ml/kg BW yielded spermatozoa with higher motility duration and Ovatide at 0.6 ml/kg BW dose was the highest among them. This is in line with Azuadi et al. (2013) who reported that Ovatide at 0.5 ml/kg of BW was the most effective hormone compared to other tested hormone. The observation of Mehdi et al. (2013) was found applicable in the present study, who stated that the motility duration depended on the dosage and method of applying hormones. This drastic reduction in motility score commensurate with the movement pattern observed with the spermatozoa and score values assigned as described by Betsy and Stephen (2014b).

Influence of higher dose of Ovatide (0.6 ml/kg BW) on the motility score and percentage of live cells also could be noticed in the present experiment. Dhawan and Kaur (2004) reported a
better performance in the fecundity and fertilization rates in *Labeo rohita* and *Cirrhinus mrigala* than those using Ovaprim in case of female fishes. The same influence in males of carps has been reported by Seifi *et al.* (2011). Similarly, in the present experiment, among the three hormones tried *viz.*, Ovatide, Wova-FH and Ovaprim, Ovatide was found to be the best in terms of motility duration, motility score and percentage of live cells leading to the conclusion that for better quality spermatozoa the synthetic hormones. Although there was negative trend or influence with regard to the density of spermatozoa due to increased doses of hormones in *C. carpio*, the quality of the spermatozoa vouch the fact that higher doses of hormones resulted in higher motility duration, that could be attributed to the higher energy spared from the higher volume of seminal plasma that was found in the milt. In the present study, it was observed that the hormonal inducements at varying doses altered the motility duration significantly. Betsy (2013) also reported that the energy in the milt could enhance the motility duration the spermatozoa of *C. carpio*.

### 5.4 Short term preservation

Short term storage of spermatozoa is general practice in artificial fertilization attempts for many fishes. Goodall *et al.* (1989) reported that fish spermatozoa are found to be active longer at lower temperatures. The effect of storage temperature on spermatozoa viability was observed by Ravinder *et al.* (1997). Freshwater fish milt has recommended to stored temperature was 0-5°C (Hulata and Rothbard, 1979). Conte *et al.* (1988) suggested that milt could be stored at 4°C. Malczewski (1988) observed that storing common carp sperm at 0-4°C up to 12 h did not reduce fertilization rate. Di Lauro *et al.* (1994) preserved sperm of Atlantic sturgeon at 4°C in plastic bags with daily replenishment of oxygen and reported 99% and 40% motility after 5 and 17 days,
respectively. The mirror carp semen can be successfully preserved for 72 h at 4˚ C prior to artificial insemination (Bozkurt and Secer, 2005).

The effect of hormonal inducement on the quality of spermatozoa of *C. carpio* was further continuously when the collected spermatozoa were subjected to short term preservation. In the present study, significant reduction in motility duration and percentage of live cells were noticed in all the samples which were subjected to short term preservation. The initial mean motility duration of spermatozoa collected after the inducement with Ovatide at a dose of 0.6 ml/kg BW decreased to 60±4.28s on 120 h indicating a mean percentage reduction of 19.0±4.97% during the short term storage periods. The percentage of motile cells was also decreased to 40% on 5th day. Similar reduction was found in treatments with Wova-FH and Ovaprim also. Reduction in the duration of motility over the period of storage at low temperature (0-5˚ C) has been reported by many authors (Kopeika *et al.*, 1997; Billard *et al.*, 2004). Therefore, the observation in the present study is a common occurrence for hormones that was found influenced by the doses of hormones.

The experiments based on the data on motility duration during short term storage reduced a sharp reduction in motility duration for the control (*R^2*=0.9793), while it was lower than 0.7 for all the hormonally induced spermatozoa. The percentage reduction in motility duration in every progressing 24 h was observed to be slower in all the samples of spermatozoa from communally induced male *C. carpio* than that of the Control. Among them the spermatozoa from the male *C. carpio* induced with Ovatidde at 0.6 ml/kg BW was the best showing lower reduction rate.

The reduction in the motility duration and percentage of live cells may be due to the anoxic condition of the milt during short term preservation. Kopeika *et al.* (1997) mentioned that
anoxic conditions encountered by the spermatozoa during storage may result in decreased ATP store in the sperm. This was confirmed by Billard et al. (2004) who reported significant decline in the ATP content in the sperm stored during in vitro storage. Szczerbowski et al. (2007) also stated that sperm which is not in contact with air will die quickly. Hence absence of oxygen supply during storage might be the result for the drastic reduction in the motility duration and percentage of live cells.

Sahin et al. (2013) reported that sperm motility and proportion of motile cells was affected as length of storage increased up to 72 h in Rainbow trout, *Oncorhynchus mykiss*. Similar results were obtained in the present experiment. The spermatozoa from hormonally induced male *C. carpio* were found to tolerate this decline in ATP, during short term storage that might be due to use of ATP in the cells or support from the higher volume of seminal plasma present in the milt, which needs to be investigated further under energetic studies of the spermatozoa.

Based on the results obtained in the present study, it could be concluded that Ovatide gave the best result in terms of motility duration as there was less motility reduction percentage over the storage of spermatozoa for short period of 120 h. It was also revealed that fishes induced with hormones at 0.6 ml/kg BW had better and higher motility durations, percentage of live cells and lowest motility reduction percentage (0.3 ml/kg BW). It was evident that the motility duration and percentage of motile cells are significantly reduced during short term preservation due to unfavorable conditions like anoxic condition which could be tolerated by the spermatozoa from the hormonally induced males *C. carpio*. Further studies with energy values of spermatozoa and its influence on enhancing the life and potency of spermatozoa during storage.
for short term to long term might be useful in designing suitable protocol for enhancing the life of spermatozoa of *C. carpio.*
6. SUMMARY

World aquaculture production is on the increasing trend. However, there is a big gap between supply and demand. Hence, there is always a need to increasing the aquaculture production through various techniques. Induced breeding of fishes was the first biotechnological tool used in aquaculture to overcome the problems faced in natural breeding. Initially natural hormones were used that was changed to synthetic hormones, due to the difficulties in collecting and preserving the natural hormones. Never the less, the influence of such hormones their doses and method of application on the quality of milt has been researched as a matter to be studied in detail.

Quality of the spermatozoa is an essential factor deciding the fertilization and survival of fry. It is necessary to study the spermatological parameters before going for artificial fertilization. Attempts to preserve the spermatozoa either for a short term or long term have been made for various species. The present work aimed to study the influence of three synthetic hormones at two different doses (0.3 and 0.6 ml/kg BW) on the spermatological qualities of Cyprinus carpio milt subjected to short term preservation.

Adult male C. carpio brooders with average body weight of 600±70g were selected based on the standard visual qualities for inducement with hormones such as Ovatide, Wova-FH and Ovaprim at doses of 0.3 and 0.6 ml/kg BW. The milt was collected after 5 hours of injection and the physical properties were observed and recorded right at the time of collection.

The volume of the milt collected from fishes induced with hormones at various doses ranged from 0.8 to 1.0 ml. The colour of milt was milky white. The pH of the milt ranged from 7.2 to 7.4.
The collected milt was diluted 40 times with 0.85% physiological saline solution. The average sperm density was $3.2\pm0.01\times10^9$ cells/ml. Control sperm density was $3.5\pm0.02\times10^9$ cells/ml. The highest mean sperm density of $4.3\pm0.25\times10^9$ cells/ml was found when Ovaprim was given at 0.3 ml/kg BW and the lowest sperm density $(1.2\pm0.1\times10^9$cells/ml) was found for Ovaprim. There was significant variation among the treatments for the spermatozoa density that was caused due to different doses of hormones administered.

The diluted milt was taken in a 10 ml beaker and covered with aluminium foil to prevent the contamination and preserved at $4^\circ$ C for 5 days. The spermatological parameters like motility duration, motility score, motility pattern, percentage of live cells and percentage reduction in the motility performance were observed and estimated at an interval of 24 h.

The initial mean motility duration in Control was $134\pm20.8$s. Among the hormonally induced samples, highest motility duration $(178\pm1.75)$ s was found when Ovatide was given at 0.6 ml/kg BW whereas, the lowest initial mean motility duration of $110\pm6.23$s was observed when Ovaprim was injected at a dose of 0.3 ml/kg BW. The motility duration of Control was reduced to $33\pm70$ s on 120h, the mean motility reduction percentage was $35.6\pm16.13\%$. There were significant differences between Control and Ovaprim at 0.6 ml/kg BW and between Control and Wova-FH at 0.6 ml/kg BW. The values were statistically significant at $P>0.01$. The percentage of live cells came down to 39% on 5th day.

The milt collected after inducement with Ovatide at 0.3 ml/kg BW displayed initial mean motility duration of $123\pm16.0$s. In 24 h, the mean motility duration was reduced to $93\pm6.12$s. After that the value decreased to $41\pm4.08$ s on 5th day of short term preservation. The initial mean motility duration of $178\pm1.75$ s was observed when Ovatide at 0.6 ml/kg BW was given.
On 120 h, of observation, the motility duration decreased to 60±4.28 s. The values were statistically significant (P>0.01). The standard mean motility reduction percentage was 19.4±2.4% and 19.0±4.97% when Ovatide was given at 0.3 and 0.6 ml/kg BW, respectively. On 5\textsuperscript{th} day, all the samples exhibited vibratory movement. The percentage of live cells was 38% and 40% in the milt samples collected after inducement with Ovatide at 0.3 and 0.6 ml/kg BW, respectively.

The initial mean motility duration was 116±10.5 s when injected with Wova-FH at 0.3 ml/kg BW which decreased to 29±6.5 s at 120 h of observation. Milt induced with Wova-FH at 0.6 ml/kg BW showed initial mean motility duration of 176±1.75 s. The value decreased to 60±4.28 s on 5\textsuperscript{th} day. The values were statistically significant between Wova-FH at 0.3 ml/kg BW and Wova-FH at 0.6 ml/kg BW and between Control and Wova-FH at 0.6 ml/kg BW (P>0.01). When Wova-FH was given at 0.3 and 0.6 ml/kg BW, the mean motility reduction percentage was 23.4±7.2% and 21.2±6.61%, respectively. The samples exhibited vibratory movement on 5\textsuperscript{th} day. The motility score of 0.5 and 0.75 with 36% and 39% of live cells were observed at 120 h when the milt samples, were induced with Wova-FH at 0.3 and 0.6 ml/kg BW, respectively.

Similarly, when Ovaprim was given at a dose of 0.3ml/kg BW, the initial mean motility duration was 110±6.23 s and it reduced to 27±1.69 s in 120h. While the initial mean motility duration was 153±6.12 s when treated with Ovaprim at 0.6ml/kg BW, the value dropped down to 100±1.24 s on 5\textsuperscript{th} day. The values were statistically significant between Ovaprim at 0.3ml/kg BW and Ovaprim at 0.6ml/kg BW and between Control and Ovaprim at 0.6ml/kg BW (P>0.01). The mean motility reduction percentage was 26.6±9.3% and 22.8±7.08% when Ovaprim was given at 0.3 and 0.6 ml/kg BW respectively. On 5\textsuperscript{th} day of observation, the samples showed
vibratory movement. When Ovaprim was given at 0.3 and 0.6 ml/kg BW, the percentage of live cells were 38% and 39%, respectively.

The present study leads to the conclusion that there are many variations between induced and non-induced spermatozoa of *C. carpio* before and after short term storage. The motility duration was higher for induced milt compared with non-induced milt. Among the three hormones tried, Ovatide gave the best result in terms of motility duration and motility reduction percentage. It was also revealed that fishes induced with hormones at 0.6 ml/kg BW had highest motility duration, percentage of live cells and lowest motility reduction percentage. Use of Ovaprim can be avoided since it showed lowest motility duration with highest motility reduction percentage.
7. REFERENCES


*Dahlgren, U., 1914. Science, **40**:862-863.


* Originals are not referred