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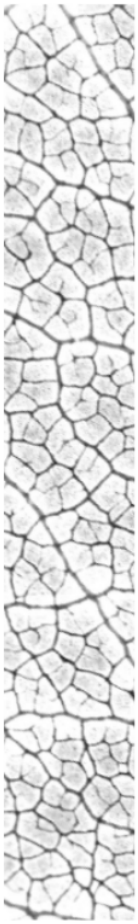

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Combination of feed protein level and laserpuncture induction of broodstock catfish (*Clarias* sp.) to increase estrogen, vitellogenin, and egg quality

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Abstract

This study determined the combined impact of various dietary protein levels (30, 35, 40%) in adult catfish feed and laserpuncture induction on enhancing the estrogen and vitellogenin levels, and egg quality, based on the fertilization rate (FR), hatching rate (HR), and survival rate (SR) after spawning. The test fish were 8–9-month-old FI hybrids obtained by cross-breeding a mature Sangkuriang female with a mature Paiton male. In total, 172 female (900–1500 g body weight) and 172 male (1140–1750 g body weight) catfish were collected from UPBAT Kepanjen District, Malang, Indonesia. Fish fed diets with increased protein levels and exposed to laserpuncture induction had significantly enhanced estrogen and vitellogenin blood serum levels ($P<0.001$) in weeks 3 and 6 compared to 6 weeks for the negative control. Based on the egg quality data, the addition of 40% protein in the diet of the reproductively mature females together with laserpuncture induction produced the highest FR, HR, and SR ($P<0.05$) compared to protein levels 30 and 35%, and the negative control.

Keywords: *Clarias* sp., estrogen, fertilization rate, hatching rate, laserpuncture induction, protein level feed, survival rate, vitellogenin

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INTRODUCTION

Sustainable catfish breeding relies on the readiness of high-quality eggs, supported by proper breeding management. However, difficulties of low egg hatchability and larvae survival rate limit the successful production of this species. Therefore, it is pivotal to optimize the factors known to affect seed production. The egg quality is highly related to the quality of catfish larvae produced (Kjørsvik et al. 1990). Although the precise determinates of egg quality are unknown, the endocrine levels during oogenesis, physical and chemical quality aspects, and quality and quantity of feed given to the adult catfish are among some of the critical, influential factors (Brooks et al. 1997, Carrillo et al. 2000).

Energy requirements during gonad maturation in catfish increase dramatically, especially during the vitellogenesis. The egg yolk composition profoundly influences egg quality because the embryo relies on the egg yolk until the larvae have learned to eat. Since the nutritional composition of the egg depends on nutrients delivered from the female, it is crucial that broodstock nutrition is optimized to support early development and good larvae survival (Izquierdo et al. 2001). A high-quality feed should contain adequate protein and essential amino acids, such as lysine and methionine, which are indispensable for catfish gonadal maturation (Pandey et al. 2004, Pandey 2013). The essential amino acids are used for sustaining life, body maintenance, growth, gonad development, and play a crucial role in endocrine function, such as estrogen production, which can improve the process of vitellogenin synthesis in the liver (Finn and Fyhn 2010, Ohkubo and Matsubara 2002). Also, amino acids also serve as the raw materials for vitellogenin synthesis, growth and follicle development in the ovaries, and egg maturation (Li et al. 2008).

In the final stages of oocyte development during vitellogenesis, the levels of gonadotropin hormone I (GtH-I), estrogen, and vitellogenin reach their peak. The oocyte then enters the maturation stage. Izquierdo et al. (2001) and Coldebella et al. (2011) stated that the adult fish feed is indispensable for the process of gonadal maturation and the spawning of silver catfish (Rhamdia

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quelen) that continuously spawn and have short vitellogenic periods. In addition to ensuring adequate protein levels in the fish feed, the gonadal maturation in fish can be accelerated by laserpuncture induction at the acupoint of female catfish reproduction.

Laserpuncture at the reproduction acupoint of broodstock catfish accelerates gonadal growth, development and maturation, as well as spawning, and procurement of catfish eggs (Kusuma et al. 2015), indicating that it may also increase the GtH-I and GtH-II (Kusuma 2013) and estrogen levels (Hariani and Kusuma 2015) in the blood. As mentioned above, a high-quality feed for adult catfish plays a vital role in producing high-quality eggs (Abidin et al. 2016). The egg quality is determined by the ability of the egg to be fertilized and then develop into a healthy embryo (Bobe and Labbe 2010, Eyo et al. 2012). The feed quality is an essential raw material for the formation of vitellogenin, a precursor for the yolk protein formation (Kapateh 2009, Kusuma and Hariani 2017, Nilsson et al. 2001). The yolk protein contributes to the development and maturation of oocytes and larvae. It is used for the maturation of a qualified egg (Hiramatsu et al. 2002a, 2002b, Muhammad et al. 2011, Sappington 2002, Yung and Show 2006), ensuring the fertilized eggs develop well by producing a high fertilization rate (FR), hatching rate (HR), and survival rate (SR).

In the study, various dietary protein levels were combined with laserpuncture induction at the acupoint of adult female catfish to enhance the estrogen level, vitellogenin, and egg quality, based on the FR, HR, and SR.

MATERIALS AND METHODS

Experimental Design, Animal Model, Acclimatization, and Treatment Conditions

This study used a nested entirely randomized design with three levels and four replications. Level I was the feed protein level (30, 35, and 40%) while Level II consisted of induction and without induction of nested laserpuncture at the protein level. Level III comprised the duration of the feed protein levels (0, 2, and 8 weeks) with and without nesting in laserpuncture induction. The parameters tested for this research were the estrogen and vitellogenin levels. Egg quality, based on FR, HR, and SR contained in two levels with four replications, was also evaluated. Level I involved the three levels of feed protein (30, 35, and 40%) while level II consisted of induced and without induced nested laserpuncture at the various feed protein levels used. The data were examined using the nested-variant analysis and continued with Duncan's multiple range test. The study was conducted for 8 months

The test fish were 8–9-month-old FI hybrids of a mature Sangkuriang female and Paiton male. A total of 172 females (900–1500 g body weight) and 172 males (1140–1750 g body weight), which had never been spawned, were obtained from one population at UPBAT Kepanjen, Malang, East Java, Indonesia. Both male and female catfish were kept in a tarpaulin pond (2 m × 2 m × 90 cm). During the 1-week acclimatization, the fish were fed formula with 30% protein content per 6% of the catfish body weight, every morning and evening. After the acclimatization, the female adult catfish placed in a tarpaulin pond each with for catfishes.

Table 1. The formulation of catfish feed used

Raw Material	Formula for each % protein level in feed (%)		
	30%	35%	40%
Fish meal	38.94	43.76	48.59
Soya flour	15.60	20.15	24.69
Comflour	10.00	10.00	10.00
Rice brain	10.00	10.00	10.00
Tapioca flour	13.46	6.92	0.37
Fish oil	3.85	2.42	1.00
Vitamin-mineral mix	2.00	2.00	2.00
CMC	6.15	4.75	3.35
Total	100.00	100.00	100.00

Table 2. Proximate analysis of the raw materials used to formulate the catfish feed

The protein level	Proximate content in the catfish feed formulation					
	Dry ingredients (%)	Ash (%)	whey protein (%)	whey fibers (%)	whey fat(%)	Gross Energy (Kkal/kg)
30%	87.35	15.88	30.15	3.69	8.07	3959.18
35%	86.7	17.21	34.72	3.78	8.56	4018.14
40%	86.45	19.31	40.05	3.89	8.62	4057.35

(Sources: proximate analysis result of the feed formulation at Nutrition Laboratory of Agricultural Faculty, Brawijaya University)

The feed formulations were prepared from fish meal, soy flour, corn flour, bran, tapioca starch, fish oil, a vitamin, and mineral premix specifically for fish, and feed adhesive material, carboxymethyl cellulose, at 30–40% protein levels (**Table 1**). The results of the proximate analysis (**Table 2**) and the amino acid contents in the raw materials used for the catfish feed formulas compared to the amino acid requirements for female catfish were analyzed (**Table 3**).

Treatment

The adult catfish with mature gonads were acclimated for 1 week and then spawned in the tarpaulin pond (1 male:1 female adult catfish). After spawning, the ovarium condition remained the same compared to the initial condition before spawning, and it was assumed that no mature eggs were present (0 weeks) (Kusuma et al. 2007). Then, the adult catfish were maintained and fed the formulated feeds containing 30, 35, and 40% protein, respectively (Sotolu 2010). Also, the catfish were induced by laserpuncture at the reproduction acupoint, specifically in the 2/3 part of the ventral body during 15 weeks, 8 weeks, and as the control used without laserpuncture induction, each week was given with one induction (Kusuma et al. 2007). The experimental treatments was carried out in 4 replications.

Table 3. The amino acids content of the catfish feed compared to the amino acids required for the female adult catfish

Amino acids composition	Amino acids required (%)	Protein level		
		30%	35%	40%
Arginin	1.8	1.71	1.99	2.28
Histidin	0.81	0.79	0.91	1.04
Isoleusin	1.29	1.05	1.21	1.38
Leusin	2.1	1.88	2.18	2.49
Lysin	2.43	2.17	2.49	2.82
Methionin	0.81	0.71	0.81	0.91
Cystein	0.27	0.28	0.32	0.36
Phenilalanin	1.23	1.05	1.23	1.40
Tyrosin	0.87	0.79	0.92	1.05
Threonin	1.35	1.23	1.42	1.61
Triptopan	0.27	0.29	0.33	0.38
Valin	1.53	1.23	1.43	1.63
Total	14.76	13.18193131	15.2553845	17.3295243
IAAE	-	89.3084777	103.356263	117.4087012

(Sources: amino acids analysis result using HPLC at Organic Chemistry Laboratory, Faculty of Mathemathics and Sciences, Gadjah Mada University)

Estrogen and Vitellogenin Measurement

Blood samples were taken from the caudal vein of the catfish body using a heparin syringe and without four heparinized syringes from each treatment. The obtained blood samples were centrifuged at 3000 rpm for 10 min at 4 ºC, and the supernatant or serum stored at -20 ºC until analysis. The estrogen and vitellogenin levels were measured using an ELISA kit (Cusabio, catalog number: CSB-E13017Fh for estrogen, and Elisa Grouper Vitellogenin (VTG) kit, catalog number: CSB-E14116Fh) (Taghizadeh et al. 2013).

Egg Quality Assessment (FR, HR, and SR)

Adult gonadal fish of each treatment were naturally located in a tarpaulin pond covered with a large *kakaban* (egg receiver). The place used as sampling size was 15 × 15 cm. Each pond was filled with two large cocoons, and each large cocoon held three small kakaban which contained fertilized eggs. The next day, the six, small hapa were taken to a hatch in a door of the pool, made of tarpaulin and equipped with heaters and 10 W lights. The eggs, laid on small cotton, were counted for the number of early eggs, fertilized eggs, and unfertilized eggs to determine the FR, as expressed in equation 1 (Mylonas et al. 2003).

$$FR(\%) = \frac{The\ total\ amount\ of\ fertilized\ eggs}{The\ total\ amount\ of\ initial\ eggs\ used} \times 100\% \quad (1)$$

After 2 × 24 h, the fertilized eggs are hatched. The hatching and not-hatched eggs were counted (HR). The eggs that were not hatched were removed from the sampling site, by using a pipette. This method was performed to calculate the HR as expressed in equation 2 (Adebayo 2006).

$$HR(\%) = \frac{Total\ number\ of\ hatched\ eggs}{Total\ number\ of\ unhatched\ eggs} \times 100\% \quad (2)$$

During days 1 to 5 after the eggs hatched, the dead and live catfish larvae were counted, to determine the SR. The dead larvae were removed from the sampling site, by using a pipette. The SR was calculated by using equation 3.

$$SR(\%) = \frac{Total\ number\ of\ live\ larvae\ after\ the\ eggs\ hatched\ (day\ 1)}{Total\ number\ of\ live\ larvae\ on\ day\ 5\ after\ the\ eggs\ hatched} \times 100\% \quad (3)$$

Data Analysis

The estrogen and vitellogenin levels and egg quality (FR, HR, and SR) were analyzed using two-way analysis of variance by GenStat version 15 software, and significant results were further evaluated by Duncan's test.

Table 4. The estrogen and vitellogenin levels in the blood serum of female catfish (*Clarias* sp.).

Treatment of protein level and laserpuncture	Mean ± deviation levels of serum estrogen level (ng/mL)	Mean ± deviation of serum vitellogenin level (ng/mL)
30% Without laser	65.495 ± 3.076^{a}	3023.888 ± 20.160^{a}
30% Laser	97.128 ± 5.143^{bc}	3537.628 ± 29.709^{a}
35% Without laser	91.48 ± 3.604^{b}	3211.460 ± 7.063^{a}
35% Laser	97.833 ± 4.731^{c}	3695.315 ± 37.838^{a}
40% Without laser	96.968 ± 1.417^{bc}	3562.815 ± 23.388^{a}
40% Laser	102.32 ± 4.057^{c}	4371.735 ± 25.781^{b}

Number followed by different letter/s (a,b, etc) indicated significantly different

RESULTS AND DISCUSSION

Estrogen Levels

The combination of feed protein levels (30, 35, and 40%) and laserpuncture induction at the reproductive acupoint of female catfish for 8 weeks, significantly ($P<0.005$) increased the estrogen and vitellogenin levels in the blood serum. The groups fed added protein (30, 35, and 40% respectively) and exposed to the laserpuncture induction at the reproductive acupoint of female catfish, yielded higher estrogen levels than the control and evidenced two estrogen peaks, at weeks 3 and 6, respectively. In contrast, the estrogen levels of the control group (without laserpuncture induction) exhibited a single estrogen peak at week 6. However, the estrogen production decreased in week 7 and fluctuated significantly ($P<0.000$) (**Table 4**).

The vitellogenin levels exhibited a similar trend to the estrogen levels. It was observed that the mean vitellogenin level was maximal at week 3 in the combined treatment groups (4371.735 ± 25.781 ng/mL) while in the negative control, it was maximal at week 6 (3562.815 ± 23.388 ng/mL). The increased levels of estrogen and vitellogenin were one indicator of the vitellogenic process in the liver. Furthermore, the results proved that the combination of the increased protein levels in the feed (30, 35, 40%) and laserpuncture induction resulted in higher levels of estrogen and vitellogenin compared to the negative control without the laserpuncture induction (**Table 4**).

The high estrogen levels in the blood serum depend on the reproduction phases of the catfish. In the fish exposed to the combined treatments (added dietary protein and laserpuncture induction), weeks 1 and 2 represented a previtellogenic stage, in which the

estrogen levels were increased. At week 3 after spawning, the estrogen level reached a peak (104 ng/mL), known as the vitellogenic stage. During weeks 4 and 5, the gonads were mature, and the estrogen levels decreased.

Week 6 was the spawning stage, and the estrogen levels in the blood serum increased significantly. Conversely, in the negative control group, the new vitellogenic stage was achieved at weeks 5 and 6. These results supported those published by Hosseinzadeh et al. (2013), who measured the estrogen levels in four stages of ovarian growth in Persian sturgeon. At the cortical alveolus stage (stage 1), the estrogen level was low (0.78 ± 0.10 ng/mL) but then peaked (5.33 ± 1.06 ng/mL) at the vitellogenic stage (stage 2). During the gonad maturation stage (stage 3), the estrogen levels declined (1.98 ± 0.48 ng/mL) and remained relatively low (2.31 ± 0.35 ng/mL) after spawning (stage 4).

The estrogen levels recorded in the catfish exposed to the combined treatment were higher than those of Hosseinzadeh et al. (2013) in Persian sturgeon fish. It suggests that the combination of additional protein in the feed with laserpuncture induction could stimulate the GtH activity, which, in turn, stimulates the gonadal gland to produce hormones associated with the reproduction system. It confirmed that supplementing the protein level in the feed combined with laserpuncture induction could stimulate the hypothalamus–pituitary-gonadal axis to release GtH. GtH-I plays an essential role in steroidogenesis, by producing estrogen and 17β-estradiol (the most potent of the naturally occurring estrogens). According to Kusuma (2013), laserpuncture induction could induce GABAergic neurons to synthesize gamma-aminobutyric acid (GABA), which relays the signal through the synaptic contacts between the nerve terminals of GABAergic neurons and gonadotropin-releasing hormone (GnRH) neurons. The released GABA further stimulates the hypothalamus to release GnRH. The GnRH neurons stimulate the pituitary to synthesize and release GtH-I into the blood, which circulates through the bloodstream to reach the gonads. GtH-I also stimulates the synthesis of testosterone that is aromatized to estrogen (17β-estradiol), catalyzed by cytochrome P450 aromatase (Diotel et al. 2010). Estrogen then stimulates the occurrence of the vitellogenic phases in the liver. Previously, in female adult catfish exposed to laserpuncture induction, the GtH-I levels increased up to 48% before the fish spawning (Kusuma 2013) and increased the estrogen levels (Hariani and Kusuma 2015). Besides, the protein content in the female adult catfish feed has been reported to influence the number of oocytes in the follicle (Lefler et al. 2008), and GtH and estrogen plasma levels (Aizen et al. 2007, Yaron et al. 2003).

Vitellogenin Level

The vitellogenin levels in the blood serum after combined laserpuncture and a high protein diet (30–40% protein content) increased during the 8-week pregnancy. The 40% dietary protein level in combination with laserpuncture induction of the female catfish resulted in higher vitellogenin levels compared to the feeds with 30 and 35% protein. It may be that combining the feeding protein content of 40% with laserpuncture induction at the reproduction acupoint of female adult catfish was able to activate peripheral nerve impulses to the recognizable brain of GABA from GABAergic neurons. GABA, which produces the female pituitary–gonad–liver–gonad on female catfish, could regulate steroidogenesis and oogenesis that begin with vitellogenesis and oocyte maturation. The critical regulator of energy in the pituitary-gonadal axis also modulates the production of reproductive hormones. GnRH stimulates the pituitary to release the GtH hormones (GtH-I and GtH-II). GtH-I is carried through the bloodstream to the gonads, where it promotes theca cells to release testosterone in large quantities. Testosterone then infiltrates the granulosa cells and is converted by P450 aromatase to 17β-estradiol (estrogen). 17β-Estradiol circulates through the bloodstream to the liver, where it promotes the biosynthesis of vitellogenin. The vitellogenin levels that were produced were dependent on the protein level in the supplied feed.

The protein present in the feed will be converted in the gastrointestinal tract of fish into free amino acids in the liver and subsequently used in vitellogenin synthesis. Increased levels of vitellogenin protein will be absorbed by the growing and developing oocytes (eggs). Fyhn (1989) demonstrated that successful embryonic development in fish is dependent on the balance of amino acids in the egg. The essential amino acids contained in the feed formula were sufficient to meet the needs of vitellogenin formation in the oocytes of the female adult catfish. It is therefore likely that adequate protein required for vitellogenin synthesis was supplied through the diet to support the egg yolk protein synthesis and thereby produce good quality eggs.

Kusuma (2013) indicated that the laserpuncture induction could increase physiological activity, such as enzyme and cell membrane activity, and also stimulate the production of reproductive hormones 3 weeks faster than without the laserpuncture induction, as the negative control. Low-power (4–5 mW) laserpuncture induction at the reproductive acupoint rapidly and directly penetrates the epidermis, dermis and, subcutaneous layers, stimulating the peripheral nerves. The electromagnetic wave energy from this laser beam is then converted into electrical signals, caused by depolarization of the nerve cell membrane, and propagated to the brain.

The release of neurotransmitters, such as GABA from GABAergic neurons, relies on cell membrane

depolarization, calcium ions, among other factors. Membrane depolarization due to the laserpuncture-induced electrical signal evokes action potential of the neuron membrane that responds with the opening of extracellular Ca^{2+} channels. The extracellular Ca^{2+} enters through the calcium-sensing receptor or voltage-gated calcium channels (Berridge et al., 2000; Clapham, 2007), causing an influx of extracellular Ca^{2+} into the presynaptic cytoplasm. Elevation of the intracellular Ca^{2+} concentration serves as a second messenger signal that triggers vesicle fusion. The vesicles then release the neurotransmitters via exocytosis into the synaptic cleft. The neurotransmitters released from the presynaptic cell then bind to specific receptors in the postsynaptic cells, eliciting either a positive (excitatory) or negative (inhibitory) effect. An excitatory postsynaptic potential means the impulse will continue until it reaches the brain, where a series of physiological reactions occur that activate glutamic acid decarboxylase (GAD-65) enzyme, which further stimulates the GABAergic neurons to synthesize GABA in brain tissue. The released GABA stimulates hypothalamic and pituitary neurons (Kusuma 2013). GABA stimulation of the hypothalamic neurons releases GnRH (Kusuma et al. 2015) that further stimulates the pituitary neurons to release GtH-I and GtH-II. The GtH-I and GtH-II are released systematically so that the levels of GtH-I and GtH-II in the blood serum increase. Studies indicate that GtH-I and GtH-II play a role in the oogenesis mechanism that stimulates the gonad glands to produce steroid hormones, like 17β-estradiol (Ohga et al. 2012, Weltzien et al. 2002). 17β-estradiol stimulates hepatic cells to synthesize vitellogenin. Muhammad et al. (2011) showed that the vitellogenin is absorbed by oocytes and deposited in a developing oocyte, and the oocyte would mature as a result of GtH-II stimulus.

The activity involved in producing estrogen and vitellogenin can occur frequently but can also be supported by high-quality feeding. Vitellogenin formed as a result of a high-protein level feed accumulates in the eggs as the main egg yolk protein (vitellin), which is then used for egg growth and maturation and assists the fish larvae activities before being fed externally. Feed formulas containing protein levels of 30–40% were therefore sufficient for vitellin synthesis in catfish. Based on the amino acid analysis, determined by high-performance liquid chromatography, the feed formula used in this study contained essential amino acids, such as arginine, histidine, isoleucine, leucine, lysine, methionine, cysteine, phenylalanine, tyrosine, threonine, valine, and tryptophan while the non-essential amino acids were glycine, serine, glutamate, alanine, and aspartate (**Table 3**). The essential amino acids present as the synthetic proteins can be used for energy in eggs, while non-essential amino acids used as the energy for the activity in the eggs.

Table 5. The mean value of fertilization rate (%), hatching rate (%), and survival rate (%) on the catfish that fed with some protein levels with and without laserpuncture induction.

Protein Levels	Laserpuncture induction*	Mean ± SD fertilization Rate	Mean ± SD hatching Rate	Mean ± SD survival Rate
30%	-	89.74 ± 0.94^{a}	92.02 ± 2.57^{a}	92.82 ± 1.31^{a}
	+	92.70 ± 1.47^{b}	95.58 ± 2.81^{b}	95.40 ±3.48^{b}
35%	-	92.21 ± 2.91^{b}	96.02 ± 2.25^{bc}	96.08 ±2.13^{bc}
	+	93.94 ± 2.19^{bc}	98.16 ± 0.35^{c}	97.09 ±1.05^{bc}
40%	-	94.19 ± 7.66^{c}	98.00 ± 7.66^{c}	97.43 ± 1.36^{bc}
	+	96.85 ± 7.66^{d}	99.19 ± 7.66^{c}	98.91 ±0.54^{c}

Note: *-, without induction; +, with induction. Number followed by different letter/s (a, b etc) showed significantly different

Egg Quality based on the Fertilization Rate (FR), Hatching Rate (HR) and Survival Rate (SR)

The combination of increased feed protein levels with laserpuncture induction at the reproduction acupoint of female adult catfish had a significant effect ($P<0.001$) on the FR, HR, and SR. Protein supplementation tended to increase all three values, particularly at the 40% dose ($P<0.05$) compared with 30 and 35% protein levels and the negative control. It was proven that the combination of protein feeding with laserpuncture induction affects the egg quality, based on FR, HR, and SR. The 30–40% protein level of catfish feed could affect the success of oocyte fertilization. The success rate of fertilization is also influenced by the quality of sperm produced by the male adult catfish. The male catfish used in this study were the offspring from hybridization of female Sangkuriang catfish with male Paiton catfish. The male catfish was maintained and fed formula with up to 30% protein, ensuring the production of good quality sperm to increase the success rate of egg fertilization (**Table 5**).

Observations by Lahnsteiner and Patemello (2002) indicated the success of fertilization was markedly influenced by the quality of the gametes and the quality of feed used. Moreover, Hariani et al. (2010) suggested that laserpuncture induction could accelerate gonadal maturation and spawning. The process of gonadal maturation and spawning is usually maintained when balanced with adequate feeding. Without adequate feeding, the total number of fertilized eggs declines.

Laserpuncture induction may stimulate follicular cell proliferation, and enzyme and hormonal activity (Karu 2000, Koutna 2003). Kusuma et al. (2015) and Kusuma and Hariani (2017) confirmed that the metabolic activity for vitellogenin synthesis and oocyte maturation in female catfish with laserpuncture induction was higher than without induction (control). However, the yolk absorption and accumulation were insufficient if the feed given to the female adult catfish was not balanced with the nutritional needs in the egg, in which case, the success of fertilization became low. Therefore, feed should contain a protein level sufficient for the formation of yolk protein. The yolk protein content inside the eggs is required to produce good quality eggs with a high FR indicator and adequate to support embryo development

(Kapateh 2009, Nilsson et al. 2001, Ohkubo and Matsubara 2002). Furthermore, the availability of the protein is pivotal, as it is needed by the female adult catfish for vitellogenesis, leading to the production of good-quality eggs.

Vitellogenin is a yolk protein precursor of fish eggs. It is a glycophospholipoprotein. The vitellogenin protein in the catfish eggs is dominated by lipovitellin. Synthesis of vitellogenin is strongly influenced by the nutrients, especially protein, which is used for yolk protein formation in the female adult catfish. During the fertilization of eggs, the vitellogenin proteins, such as lipovitellin and phosvitin, rapidly degrade into free amino acids and free fatty acids that are used as an energy substrate and for protein synthesis in embryonic development. Carnevali et al. (2006) suggested that during the embryonic development, the rapid degradation of lipovitellin and phosvitin produced free amino acids, free fatty acids, phosphorus, and calcium. Afkhami et al. (2011) confirmed that the free amino acids in yolk are essential for the embryo and larvae development. Hiramatsu et al. (2002a) suggested that lipovitellin is a significant source of nutrients for embryo development because it is used directly for embryonic tissue synthesis and as a source of energy for embryonic metabolism.

The quality of the protein feed was determined by its amino acid composition. Amino acids are one of the egg yolk biochemical components that play a vital role in the maintenance, growth, and the reproduction of fish. Besides the protein components, including amino acids, the yolk contains lipids, fatty acids, carbohydrates, and vitamins. Among these nutrients, lipids and fatty acids are the primary energy sources for fish while proteins are essential in larval development and growth. The biochemical composition in the egg yolk of the catfish fed with 30–40% dietary protein included essential amino acids, ie. lysine and methionine, required for the vitellogenin formation in the egg that supports the many physiological activities that are essential for the early stages of embryogenesis. Furthermore, the excess protein could be utilized for the growth, development, and maturation of eggs, and embryonic development until the larvae was 5 days post-hatching. Lipids, carbohydrates, vitamins, and minerals contained in the feed formula remain necessary for embryonic development. Pandey (2013) agreed that adult catfish fed with high nutritional feed could affect the quality of gametes and seeds.

Thus, the content of amino acids in the egg yolk determined the quality of the egg, which, in turn, was influenced by the quality of the feed. The supply of amino acids inside the egg yolk is depleted during fertilization and embryonic development (Farhoudi et al. 2012). Mejri and Rejean (2014) noted that the lysine and serine content in the eggs increased during spawning and were preferentially exhausted during embryogenesis. Naz (2009) confirmed that during fertilization, utilization of leucine and alanine was very high in the gilthead seabream (Sparus aurata L.) eggs.

Vitellogenin is a precursor of yolk protein, a glycophospholipoprotein. The vitellogenin dominated in the catfish known as lipovitellin. The vitellogenin synthesis requires high-quality containing protein feed for the female adult catfish to produce good-quality yolk protein. During fertilization, the vitellogenin proteins, such as lipovitellin and phosvitin, are rapidly degraded into free amino acids and free fatty acids, which are used for energy and protein synthesis during the embryonic development stage. Carnevali et al. (2006) mentioned that rapid degradation of lipovitellin and phosvitin occurred during the embryonic development, resulting in the production of free amino acids, free fatty acids, phosphorous and calcium. Afkhami et al. (2011) suggested that the free amino acids in yolk were essential for the embryos and larvae development. Hiramatsu et al. (2002a) indicated that lipovitellin could be used as a primary source of nutrition for embryo development, directly used for embryonic tissue synthesis, and also used for embryonic metabolism energy. The metabolic energy used for embryo development was inseparable from the feed quality that given.

Samaee et al. (2010) stated that arginine and glutamine contribute to fertilization. Mejri and Rejean (2014) observed that arginine, phenylalanine, histidine, lysine, glutamic acid, and aspartic acid were the dominant amino acids in the egg yolk of *Acanthopagrus latus* fish after fertilization while alanine and tyrosine were at the lowest levels. Catfish fed a high nutritional feed containing protein was necessary for the vitellogenin formation, which can be used as a determinant for the high fertilized eggs, and the laserpuncture induction was also essential to speed up the maturation and spawning process in the catfish. The combination of feed containing a certain level of protein and the laserpuncture induction was proven to increase the FR. However, the FR is also influenced by the environmental conditions. Temperatures between 27.30-27.60 °C were optimal in this study for catfish spawning. The temperature may affect the duration of hatching. Islam (2005) noted that the hatching of Pangasius sutchi was inversely correlated with the temperature fluctuations. At 26 °C, hatching of P. sutchi typically occurs within 24 h after fertilization. However, at 20 °C, it took 32 h after fertilization.

Based on the HR, the current study demonstrated that the combination of increased protein level in the feed with laserpuncture induction affected the egg quality. The HR reflects the quality of successfully fertilized oocytes. In this study, the HR was > 90%. According to Samaee et al. (2010), egg quality can be categorized as high (HR > 72%), medium (HR > 33–72%), or low (HR < 3%). Adewumi et al. (2005), Bobe

and Labbe (2010) and Eyo et al. (2012) indicated that increasing the protein level in catfish feed significantly impacted egg and sperm quality, as demonstrated by a high FR and HR. Therefore, preparation of the feed formula should consider the nutritional needs of the eggs, which are inseparable from the nutritional needs of protein quality. Degradation of animal protein provides amino acids for glucogenesis in the liver (Li et al. 2008, Pandey et al. 2004, Singh et al. 2012) and is an important substrate in fish feed, to accelerate gonad maturation and improve the quality of the gametes. Samaee et al. (2010) recognized that glutamic acid had a close association with the HR on day 0 after hatching. Naz (2009) found that after egg hatching until 96 h, the content of amino acids, such as histidine, lysine, leucine, and alanine, in the yolk sac decreased. Finn and Fyhn (2009) suggested that the embryo and fish larvae can use amino acids in yolk sacs as the catabolic substrates for intermediary metabolism. About 48% of non-polar amino acids, such as glycine, alanine, proline, valine, isoleucine, and leucine, contributed to the function of lipoproteins in the transportation of endogenous lipids (Finn and Fyhn 2009).

The embryo and larvae also utilize lipoproteins and phosvitins. Lipovitellin and phosvitin are the main constituents of vitellogenin. As pointed out by Prakash et al. (2013), the lipovitellin in the yolk provides lipoproteins, and the high serine content phosvitin is the principal source of phosphoproteins. Twenty percent or more of the lipovitellin structure was lipid while phosvitin accounted for 3% of the total yolk protein (Prakash et al. 2013).

The SR success was highly determined by the protein level of the catfish feed and nutrient reserves in the yolk sac. The higher protein levels in feed proved to produce higher SR than other protein levels. This outcome was attributed to the high larval survival, demonstrating the egg yolk reserves were sufficient, until the larvae were fed exogenously. It is known that free amino acids produced from proteolysis of vitellogenin can be used as an energy source for the larvae growth and development (Carrillo et al. 2000, Finn 2007, Ohkubo and Matsubara 2002). Furthermore, the amino acids, alanine, and aspartate, in eggs have been demonstrated to be the main glucogenic precursors, supplying energy for the larvae (Li et al. 2008). Samaee et al. (2010) mentioned that the total content of free amino acids, such as glutamic acid, asparagine, glutamine, and arginine, correlated with the larvae viability. The viability of larvae depends on the biochemical composition of the egg, as it may be illustrated the embryonic needed for nutrients associated with larval growth and development.

The SR was highly dependent on the egg yolk quality and contents during embryogenesis, and the developing larvae utilized the yolk availability after fertilization and hatching until 5 days post-hatching. It was important that the availability of yolk in the eggs was dependent on the feeding protein level of the adult catfish. Therefore, in the manufacture of feed formulas for the adult catfish should refer to the yolk amino acids content in the eggs. Finn and Fyhn (2009) suggested that embryo development and fish larvae relied on the egg yolk amino acids as a catabolic substrate in intermediary metabolism and as an anabolic substrate for protein synthesis. According to Abboudi et al. (2006), the essential amino acids in the egg yolk are utilized by the fish larvae to grow while non-essential amino acids are used as the energy substrate. It has been previously shown that temperature (Kelly 2004, Oyelese 2006, Puvaneswari et al. 2009) and free amino acids, such as glutamic acid and asparagine (Samaee et al. 2010), contribute to the HR and SR during the post-hatch larvae development. The optimal temperature for egg incubation is reportedly 26–30 ºC because it is characterized as accelerating larvae development (Kelly 2004, Oyelese 2006). Under this condition, Puvaneswari et al. (2009) observed complete yolk sac absorption at 3 days post-hatching, indicated by morphological changes in the larvae.

As mentioned above, the cellular activity during catfish larvae development is influenced by the temperature and feed protein level. Izquierdo et al. (2001) and Akankali et al. (2011) stated that sufficient temperature and nutritional reserves within the egg yolk significantly affected the larval survival after hatching. In the current study, high egg quality was obtained due to the combination of protein levels in the catfish feed and the laserpuncture induction. The findings highlighted that laserpuncture induction could accelerate the egg maturation, and the accumulation of yolk protein was sufficient for metabolic activity during development of the fertilized eggs, resulting in a high FR, HR, and SR. The contribution of protein from the high-quality feed supported the metabolic activity in the acceleration of the egg maturation process due to laserpuncture induction, and the yolk protein accumulation was indispensable for the catfish embryo development. Thus, the yolk protein served as a sufficient food reserve for embryo development before the hatching of the fertilized eggs and larvae development until day.

Based on result and discussion, combination of 40% protein in the feed combined with laserpuncture induction increased the estrogen and vitellogenin levels in the blood serum and improved the egg quality, based on FR, HR, and SR. It was suggested to use the combination of 30% protein level in the feed and the laserpuncture induction at the reproduction acupoint, to improve the egg quality.

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