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Luteinizing Hormone is an effective replacement for hCG to induce ovulation in *Xenopus*

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ABSTRACT

Injection of human Chorionic Gonadotropin (hCG) directly into the dorsal lymph sac of *Xenopus* is a commonly used protocol for induction of ovulation, but recent shortages in the stocks of commercially available hCG as well as lack of a well tested alternative have resulted in frustrating experimental delays in laboratories that predominantly use *Xenopus* in their research. Mammalian Luteinizing Hormones (LH) share structural similarity, functional equivalency, and bind the same receptor as hCG; this suggests that LH may serve as a good alternative to hCG for promoting ovulation in *Xenopus*. LH has been found to induce maturation of *Xenopus* oocytes in vitro, but whether it can be used to induce ovulation in vivo has not been examined. Here we compared the ability of four mammalian LH proteins, bovine (bLH), human (hLH), ovine (oLH), porcine (pLH), to induce ovulation in *Xenopus* when injected into the dorsal lymph sac of sexually mature females. We find that both ovine and human LH, but not bovine or porcine, are good substitutes for hCG for induction of ovulation in WT and J strain *Xenopus laevis* and *Xenopus tropicalis*.

1. Introduction

Induction of ovulation in *Xenopus* by injection of human Chorionic Gonadotropin (hCG) directly into the dorsal lymph sac is a well established protocol (Sive et al., 2000). This protocol has its roots in some of the earliest experimental work done in *Xenopus* more than eight decades ago. In the early 1930s, while studying the role of pituitary hormones in *Xenopus laevis* pigmentation, Hogben et al. (1931) described that injection of extracts from the anterior pituitary of an ox could induce ovulation in *X. laevis* at any time of the year and that hypophysectomy resulted in striking ovarian regression. This suggested that *X. laevis* may serve as a good system for detection of gonadotropic activity and directly led to the development of the *Xenopus* test for early pregnancy in which induction of ovulation was scored following injection of urine collected from human females (Elkan, 1938, 1946). Further work in biochemistry led to the purification and identification of hCG as the active component in pregnant urine responsible for induction of ovulation in the frog and eventually led to commercial availability of purified hCG (Practice Committee of American Society for Reproductive Medicine, Birmingham, Alabama, 2008). The increased access to the purified hormone together with development of protocols for maintenance and breeding of captive *X. laevis* allowed for its establishment as an experimental model system capable of providing large quantities of equivalent material all year round for use in biochemistry, embryology, and development biology (Gurdon and Hopwood, 2000).

Although Chorionic Gonadotropin (CG) is only found in primate and equine genomes, it is structurally similar to Luteinizing Hormone (LH), which is found in all vertebrates (Choi and Smitz, 2014). Both CG and LH are heterodimer glycoproteins composed of two subunits, α and β (Choi and Smitz, 2014); the α subunit is identical for both, while the β subunits are encoded by distinct genes (Boorstein et al., 1982; Naylor et al., 1983). In vivo, each hormone exists as a cocktail of distinct isoforms resulting from extensive post-translational glycosylation, sialylation and sulphonation (Choi and Smitz, 2014). The differences in the extent of post-translation modification between the two hormones are thought to account for the variability in their perdurance and for the fact that despite both activating the same Luteinizing Hormone/choriogonadotropin receptor (LHCGR), each promotes a distinct downstream signaling response (Choi and Smitz, 2014).

In recent years, the intermittent shortages of commercially available hCG have led to sporadic and frustrating interference with experimental work relying on induction of *Xenopus*

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ovulation. In spite of the described differences between the two hormones, previous data using *Xenopus* oocytes and other anuran species suggest that LH derived from mammalian sources is a good candidate as an alternative to hCG in induction of *Xenopus* ovulation. In amphibians, the final steps of oocyte maturation occur shortly before ovulation and are characterized by meiotic division and the resulting breakdown of nuclear envelope and extrusion of the first polar body (Thornton, 1971). Ovine LH (oLH) was demonstrated as highly specific in promoting oocyte meiotic maturation in isolated *X. laevis* ovaries (Licht et al., 1976). Furthermore, oLH effectively induced ovulation in ovaries isolated from another anuran, *Rana pipiens* and also stimulated high levels of progesterone production in ovarian fragments from *Rana catesbeiana* (Bergers and Li, 1960; Licht et al., 1976; Ogawa et al., 2011).

We decided to investigate whether injection of mammalian LH proteins into the dorsal lymph sac of *X. laevis* and *X. tropicalis* would be sufficient to promote ovulation and therefore could be used as an alternative to hCG. We tested the efficiency of LH proteins derived from four distinct mammalian sources: bovine (bLH), human (hLH), ovine (oLH) and porcine (pLH) to induce egg laying in *Xenopus* by measuring the number of eggs laid. With the recent completion of the *X. laevis* genome using the inbred J strain (Session et al., 2016) we compared the responses in both outcrossed wild type (WT) and inbred J strain *X. laevis*. We found that oLH and hLH are as efficient at inducing spawning as hCG, whereas bLH and pLH were less effective at inducing ovulation. In *X. tropicalis*, we found that oLH is as efficient as hCG in inducing ovulation, but that the batch size produced by oLH injected females is always smaller. Nonetheless, the oLH injected *X. tropicalis* females still produce eggs in numbers sufficient for experimental work. Our results demonstrate that oLH and hLH can be used as a substitute for hCG in *Xenopus* to promote ovulation.

2. Materials and methods

2.1. Husbandry

All animals were housed and handled in the National *Xenopus* Resource in accordance with animal care protocol 15-02B approved by the Marine Biology Laboratory IACUC.

2.2. Spawning

Spawning was induced in sexually mature *X. laevis* wild type and J strain females by first priming with an injection of 50 IU (WT) or 35 IU (J strain) PMSG, respectively, followed 2–4 days later by the injection of hCG, recombinant hCG, bovine LH, human LH, ovine LH, or porcine LH. Two females per tank were kept at room temperature and were allowed to spawn for 22 h. The next day each female was squeezed 2–3 times to help induce spawning. After 22 h, all eggs were collected and the volume of eggs with jelly coats still on was measured and used to estimate the total number of eggs. Eggs were collected into falcon tubes and allowed to settle for at least 5 min before volume measurements were made.

A similar approach was used for *X. tropicalis* where females were primed with 10 IU of hCG or 15 IU of PMSG, then boosted 1–2 days later with hCG, rhCG, or oLH. Following boosting, females were kept at 3 per tank at 25–27 °C and allowed to lay eggs for 8 h, after which the eggs were collected, their volumes measured and the average number of eggs laid per female was calculated. While laying, each female was squeezed 1–2 times.

Females that did not produce any eggs during the squeezing nor during frequent observations throughout the experiment as well as the ones that did not show a pronounced engorgement of the labia and as such were not responding to the hormone were considered as not having laid any eggs throughout the experiment.

2.3. Egg counting

To convert volume of eggs laid to total number of eggs we first established the average number of eggs per mL laid by J strain and WT *X. laevis* and *X. tropicalis* as follows. 5 mL of eggs were collected in a 50 mL falcon tube from three individual females and, after dejellying, the total number of eggs was counted and used to calculate the average number of eggs/mL. This gave 150 eggs/mL for WT *X. laevis*, 200 eggs/mL for J strain, and 344 eggs/mL for *X. tropicalis*.

2.4. Hormones

Highly purified bovine, human, ovine and porcine Luteinizing Hormones as well as rhCG and hCG were ordered from the National Hormone and Peptide Program (www.humc.edu/hormones). PMSG was procured from Fisher Scientific (Catalog # 50893505).

2.5. Statistical analysis

Statistical analysis was performed in MATLAB. The following code was used.

```
hcg=[2775 3100 3900 3800 500 6075 975 8250];
lh=[4900 3800 2500 3300 3600 9200 400 2200 2700 7200 4050];
[h, p, ks2stat]=kstest2(hcg, lh).
```

3. Results

3.1. Efficient induction of ovulation in Xenopus with ovine Luteinizing Hormone

To determine if oLH can be used as a substitute for hCG, we compared the ability of each hormone to induce ovulation in vivo in *X. laevis* in both inbred J strain and outcrossed female *X. laevis*. Initially, we used oocyte positive females (i.e. those with a demonstrated prior history of oocyte production) that were at least 18 months old. As J strain frogs are smaller than traditional outcrossed frogs we tested various doses of each hormone. Female frogs were initially primed with PMSG 2–4 days prior to injection of hCG or LH and were not fed during this time. The frogs were then injected with different doses of hormone and allowed to lay eggs for 22 h, after which the eggs were collected and the volume measured; during egg laying, the frogs were manually squeezed three times before collection. At 350 IU, 400 IU, or 450 IU of hCG female J strain frogs produced approximately 2400 eggs (Fig. 1A). At the highest dose of 500 IU of hCG J strain female frogs produced an average of 3294 eggs (Fig. 1A). In comparison, J strain females also responded well to oLH. At the lowest dose (100 μg) the females laid fewer eggs than hCG, averaging 1880 eggs (Fig. 1A). The number of eggs laid increased with higher doses of oLH; at 150 μg they produced 2888 eggs, and females boosted with 200 μg oLH produced 3738 eggs (Fig. 1A). Although all three doses were sufficient to induce egg laying, the highest dose of oLH was the most efficient and consistent. All eggs laid by J strain females were of good quality and fertilized efficiently in vitro with J strain male sperm and developed normally (data not shown). We have been using oLH in place of hCG for over six months now and have not seen a large difference in quality of eggs produced and fertilized, nor has this affected the maturation of resulting tadpoles to froglets and adults.

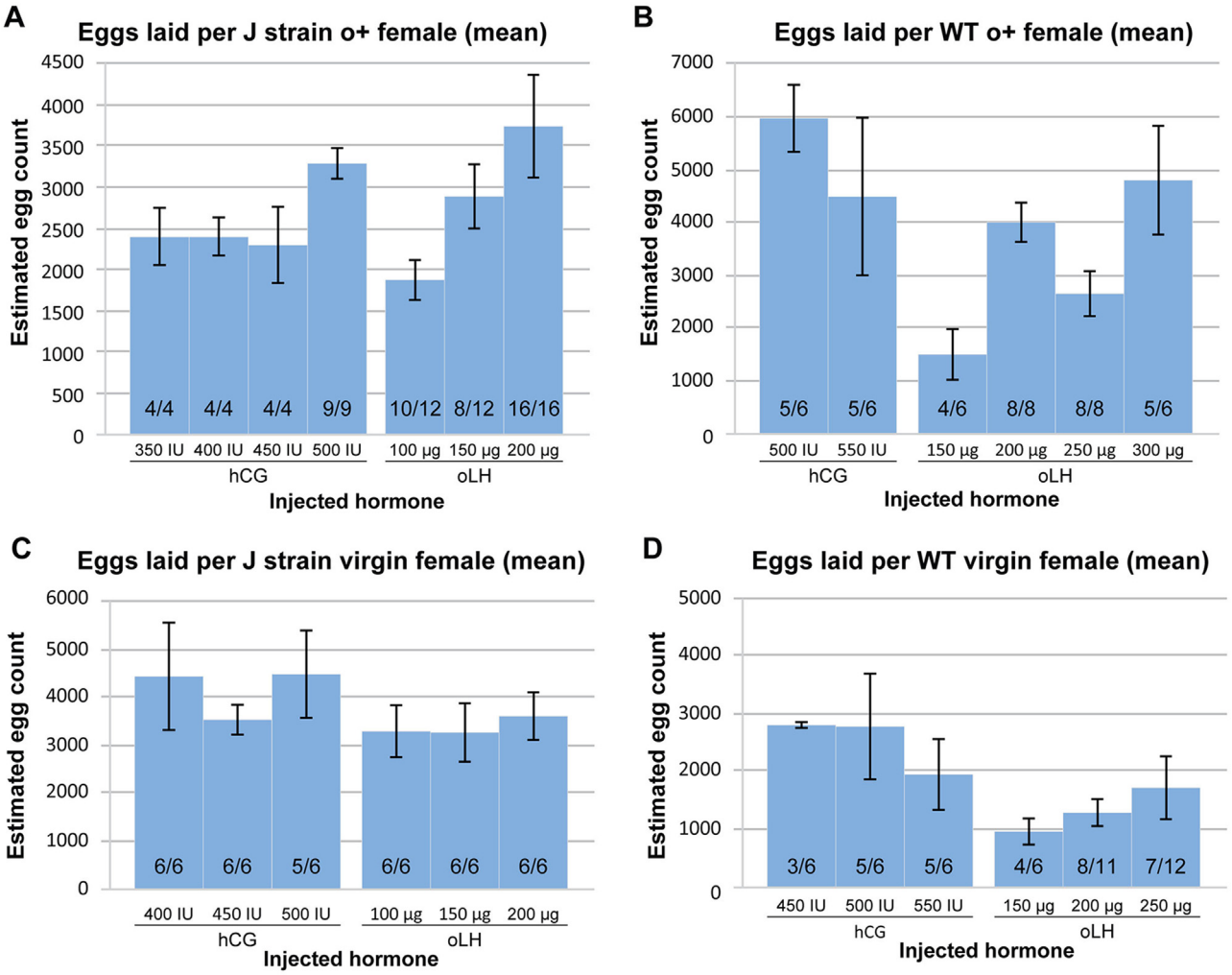

Fig. 1. Ovine Luteinizing Hormone efficiently induces spawning in both J strain and WT *X. laevis*, independent of egg laying history. (A) J strain o+, (B) WT o+, (C) J strain virgin, and (D) WT virgin *X. laevis* females were boosted with either hCG or oLH. The height of each bar indicates the average number of eggs laid per female with the error bars showing the standard error of the mean. The ratios within each bar represent the corresponding number of females that laid eggs per number of females boosted. Only females that laid were included in the calculations of average number of eggs laid and the standard error of the mean. The amount and type of hormone used are indicated on the bottom of each graph.

The inbred J strain is of particular importance due to its use for the sequencing of *X. laevis* genome (Session et al., 2016); since many laboratories working with *X. laevis* predominantly use outcrossed wild type (WT) frogs purchased from commercial vendors we examined whether oLH was as effective at inducing ovulation in WT *X. laevis*. At 500 IU and 550 IU of hCG the females produced an average of 5940 and 4470 eggs (Fig. 1B); lower doses of hCG were not effective at inducing egg laying in WT *X. laevis*. We observed a comparable spawning efficiency at the highest doses of oLH used. At 150 µg oLH the boosted animals produced an average of only 1500 eggs, with only 66% of injected frogs laying eggs (Fig. 1B). At higher doses we found more consistent numbers of WT frogs laying eggs; at 200 µg they produced 3994 eggs, at 250 µg they produced 2644 eggs, and at the highest dose of 300 µg they produced 4700 eggs (Fig. 1B). Thus, oLH is able to induce spawning in oocyte positive outcrossed females at efficiencies comparable to those obtained with hCG, but required slightly higher doses as compared to J strain females. This is consistent with the fact that WT o+ frogs are approximately 60% larger than J strain frogs (Table 1).

Both hCG and oLH efficiently induce ovulation at the highest dose tried. Combining the results from J strain and WT oocyte positive females, 14 females of the 15 injected with hCG and 21 of the 22 injected with oLH ovulated. In both conditions, a single WT female did not spawn. High rates of ovulation do not necessarily mean that both hormones are able to induce production of equally high numbers of eggs. To test the equivalency of the two hormones in relation to the number of eggs produced we performed a two-sample nonparametric Kolmogorov-Smirnov test (Massey, 1951). In our experimental design two females were included per tank and the number of eggs laid was calculated as an average and thus to provide sufficient power for this statistical analysis we combined the J strain and WT data. The tanks that included the WT females that did not lay were also included in the calculation. We were not able to reject the null hypothesis that both hormones would produce equivalent egg number distributions and the asymptotic P-value (considered accurate for our sample sizes) is 0.9183. This supports the conclusion that at the highest doses tested not only are hCG and oLH essentially identical in their efficiency at inducing ovulation but they also produce equivalent numbers of eggs.

To assess whether prior spawning history had any influence on the ability of *X. laevis* females to respond to oLH, we compared the efficiencies of hCG and oLH to promote egg laying in virgin J strain and WT females. In virgin J strain females boosted with hCG we found similar results with all three doses tested. At 400 and 450 IU

Table 1
Weight of *X. laevis* and *X. tropicalis* and associated hormone dose.

Figure panel	Strain	Breeding history	Experimental condition	Individual mass of females used (g)	Mean mass (g)	Hormone dose per gram mass
1A	J	O+	350 IU hCG	58.2, 63.9, 49.3, 57.0	57.1	6.13 IU
			400 IU hCG	76.9, 66.1, 79.7, 83.7	76.6	5.22 IU
			450 IU hCG	89.7, 72.3, 53.2, 52.9	67.03	6.71 IU
			500 IU hCG	59.0, 80.0, 68.0, 70.0, 60.0, 80.0, 55.7, 91.6, 78.3	71.4	7.00 IU
			100 μg oLH	58.9, 74.6, 72.2, 66.8, 42.5, 52.2, 42.4, 74.0, 101.0, 84.5, 105.0, 96.0	72.53	1.38 μg
			150 μg oLH	66.0, 55.2, 56.2, 69.5, 90.0, 113.5, 94.4, 107.0, 86.0, 95.0, 79.3, 99.2	84.28	1.78 μg
			200 μg oLH	87.0, 72.0, 85.0, 69.0, 92.1, 75.8, 57.8, 74.3, 95.0, 93.5, 97.0, 106.5, 80.1, 70.2, 74.0, 89.0	82.39	2.43 μg
1B	WT	O+	500 IU hCG	79.4, 98.3, 88.5, 75.8, 82.5, 152.4	96.15	5.20 IU
			550 IU hCG	88.2, 77.6, 63.3, 174.5, 171.2, 112.9	114.62	4.80 IU
			150 μg oLH	97.3, 135.8, 130.4, 93.9, 109.7, 154.3	120.23	1.25 μg
			200 μg oLH	110.4, 139.4, 99.3, 140.4, 171.8, 203.1, 164.5, 259.3	161.03	1.24 μg
			250 μg oLH	126.0, 85.0, 88.7, 87.9, 129.2, 144.4, 145.0, 170.4	122.08	2.05 μg
			300 μg oLH	121.9, 87.8, 137.6, 138.5, 124.1, 195.5	134.23	2.24 μg
1C	J	Virgin	400 IU hCG	52.2, 43.3, 62.0, 64.4, 55.5, 34.2	51.93	7.70 IU
			450 IU hCG	49.7, 52.2, 48.7, 53.4, 60.9, 34.1	49.83	9.03 IU
			500 IU hCG	61.4, 72.1, 64.4, 53.4, 49.7, 41.1	57.07	8.76 IU
			100 μg oLH	75.3, 59.5, 49.6, 60.1, 39.6, 39.8	53.98	1.85 μg
			150 μg oLH	61.5, 74.2, 50.9, 71.1, 33.3, 69.8	60.13	2.49 μg
			200 μg oLH	56.6, 74.5, 55.5, 72.6, 65.4, 48.3	62.15	3.22 μg
1D	WT	Virgin	450 IU hCG	73.0, 86.1, 88.3, 98.6, 96.4, 97.7	90.02	5.00 IU
			500 IU hCG	112.8, 131.5, 104.1, 137.8, 73.4, 123.9	113.92	4.38 IU
			550 IU hCG	108.2, 85.4, 97.4, 114.5, 52.1, 87.8	90.9	6.05 IU
			150 μg oLH	105.9, 105.4, 108.1, 115.6, 96.4, 126.4	109.63	1.37 μg
			200 μg oLH	143.9, 99.8, 110.5, 87.2, 82.4, 81.3, 146.8, 118.5, 82.8, 93.0, 104.3	104.58	1.91 μg
			250 μg oLH	108.5, 135.1, 102.5, 113.8, 104.0, 120.0, 76.5, 128.8, 61.6, 92.5, 87.0, 91.6	101.81	2.46 μg
2A	J	O+	100 μg bLH	55.2, 76.5, 63.4, 82.1, 87.5, 32.9	66.27	1.51 μg
			150 μg bLH	61.3, 41.3, 82.5, 29.9, 47.4, 88.1	58.43	2.57 μg
			200 μg bLH	95.9, 87.7, 47.5, 49.6, 53.0, 77.6	68.54	2.92 μg
			100 μg hLH	67.4, 55.1, 57.0, 60.1, 99.2, 77.3	69.35	1.44 μg
			150 μg hLH	58.4, 69.7, 93.6, 98.5, 69.1, 53.4	73.78	2.03 μg
			200 μg hLH	49.6, 100.5, 97.0, 67.0, 47.9, 48.2	68.37	2.93 μg
			100 μg pLH	44.4, 68.7, 65.2, 92.5	67.7	1.48 μg
			150 μg pLH	48.2, 91.6, 57.4, 74.2, 70.8, 61.9	67.35	2.23 μg
			200 μg pLH	62.6, 107.9, 64.8, 87.7, 61.0, 85.6	78.28	2.56 μg
2B	WT	O+	200 μg bLH	164.3, 124.4, 154.1, 107.0, 164.4, 170.8	147.5	1.36 μg
			250 μg bLH	124.0, 73.8, 85.2, 157.0, 93.6, 200.3	122.32	2.04 μg
			200 μg hLH	129.8, 127.3, 85.0, 132.0, 153.3, 215.2	140.43	1.42 μg
			250 μg hLH	157.6, 125.3, 80.3, 168.7, 106.6, 151.3	131.63	1.90 μg
			200 μg pLH	139.4, 192.7, 165.9, 205.8, 86.2, 112.4	150.4	1.33 μg
			250 μg pLH	171.6, 184.5, 116.5, 166.1, 170.4, 178.9	164.67	1.52 μg
3	TROP	O+	10 IU hCG/100 IU hCG	18.1, 23.5, 16.9, 19.9, 17.0, 18.9	19.05	5.25 IU
			10 IU hCG/150 IU hCG	15.1, 23.2, 17.4, 23.2, 17.2, 13.6	18.28	8.20 IU
			10 IU hCG/100 IU rhCG	16.8, 23.2, 17.9, 14.5, 16.5, 12.6	17	5.88 IU
			10 IU hCG/150 IU rhCG	15.6, 20.8, 23.4, 19.7, 13.2, 14.2	17.82	8.42 IU
			10 IU hCG/25 μg oLH	16.0, 14.4, 17.7, 17.1, 11.4, 16.3	15.48	1.62 μg
			10 IU hCG/50 μg oLH	20.3, 16.7, 17.1, 17.4, 16.0, 18.1, 15.9, 20.7, 15.9, 19.2	17.73	2.82 μg
			10 IU hCG/75 μg oLH	14.3, 14.5, 13.6, 14.7, 17.7, 19.8, 15.8, 12.1	15.31	4.90 μg
			10 IU hCG/100 μg oLH	17.9, 13.6, 14.5, 18.7, 11.6, 16.4, 15.9	15.51	6.45 μg
			15 IU PMSG/100 IU hCG	19.0, 16.6, 21.6, 17.9, 13.0, 15.4	17.25	6.80 IU
			15 IU PMSG/150 IU hCG	14.0, 21.1, 21.5, 17.4, 16.8, 15.5	17.72	8.47 IU
			15 IU PMSG/100 IU rhCG	17.8, 24.6, 20.0, 16.3, 16.7, 18.5, 18.6	18.93	5.28 IU
			15 IU PMSG/150 IU rhCG	13.9, 18.8, 16.5, 16.1, 15.2, 14.2, 16.3	15.86	9.46 IU
			15 IU PMSG/25 μg oLH	15.7, 18.0, 11.6, 17.9, 14.3, 16.9, 18.1, 15.5	16	1.56 μg
			15 IU PMSG/50 μg oLH	19.2, 15.5, 16.0, 17.1, 15.7, 10.0, 13.8, 19.6, 16.7, 17.2	16.08	3.11 μg
			15 IU PMSG/75 μg oLH	13.2, 17.3, 14.2, 15.7, 19.4, 15.1, 14.9, 11.1, 12.7	14.84	5.05 μg
			15 IU PMSG/100 μg oLH	15.4, 20.2, 15.4, 17.7, 13.3, 17.3, 17.7, 19.3, 20.6	17.43	5.74 μg

all six female frogs laid an average of 4433 and 3533 eggs, respectively, while five out of six females injected with 500 IU produced an average of 4480 eggs (Fig. 1C). Boosting virgin J strain females with oLH was also effective with all virgin frogs tested laying eggs; 3300 eggs with 100 μg, 3267 with 150 μg, and 3600 with 200 μg (Fig. 1C). Conversely, in WT virgin females we found that oLH was less effective than hCG at inducing egg laying; most hCG injected virgin females laid eggs, whereas the results were more variable with oLH. hCG injected virgin females laid an average of 2800 eggs when injected with 450 IU (3/6), 2775 with 500 IU (5/6), and 1950 with 550 IU (5/6) (Fig. 1D). oLH injected virgin WT frogs laid fewer eggs than with hCG, even at the highest

dose of oLH. At 150 µg four of six females laid 975 eggs, 1294 eggs were laid by eight of eleven females injected with 200 µg, and at 250 µg the females produced 1714 eggs (Fig. 1D). Thus, in virgin female frogs hCG was more effective at inducing egg laying and produced a greater number of eggs than oLH.

Based on our results we found that oLH is an effective substitute for hCG, and the most effective dose correlates with the size of the female frog. Overall, we found J strain frogs weigh approximately 60% less than WT frogs, with J strain virgin frogs averaging 55.8 g and J strain o+ frogs weighing 76.2 g, whereas WT virgin frogs were on average 102.1 g and WT o+ frogs weighing 126.5 g (Table 1). To determine the most effective dose we calculated the amount of oLH used (µg) per gram body weight that resulted in the largest number of eggs and highest percentage of injected females laying. In the oLH injections the hormone concentrations tested ranged from 1.38 µg to 3.22 µg per gram of average body mass in J strain frogs, and from 1.25 µg to 2.46 µg per gram of body mass in WT frogs (Table 1). Based on these results we estimate that 2.5 µg/g of oLH is the most effective dose; on average this amounts to 150–200 µg for J strain frogs and 250–300 µg for WT frogs.

3.2. *Human but not bovine nor porcine Luteinizing Hormones promote efficient egg laying in X. laevis*

The amino acid sequences of Luteinizing Hormone in various species are highly similar, but contain different posttranslational modifications. We decided to test whether using Luteinizing Hormone derived from three alternate mammalian sources, bovine (bLH), human (hLH), and porcine (pLH), would demonstrate a similar ability to promote egg laying in *X. laevis* as oLH and hCG did. For consistency, we tested the effectiveness of other LH preparations only in o+ J strain and WT frogs and found comparable results in both strains. In J strain females, bLH was an inconsistent inducer of egg laying, with only 300 eggs produced by two of six female frogs injected with 100 µg, 1950 eggs laid by four of six injected with 150 µg, and 3500 eggs laid by only two of six injected with 250 µg (Fig. 2A). hLH was considerably more effective at inducing spawning with 100 µg producing an average of 3200 eggs, 150 µg producing 4500 eggs, and 200 µg producing 3456 eggs (Fig. 2A). Boosting with pLH resulted in poor and inconsistent spawning with none of the four females injected with 100 µg producing any eggs, only two of six injected with 150 µg producing an average of 1400 eggs, and two of six injected with 200 µg producing an average of 500 eggs (Fig. 2A).

WT females showed somewhat similar responses to these three mammalian hormones. They responded more consistently to bLH but produced small egg batches. Four of six injected with 200 µg of bLH produced an average of 1706 eggs, and all six injected with 250 µg produced 2050 eggs on average (Fig. 2B). hLH was more efficient at inducing spawning. All six females injected with 200 µg laid an average of 3756 eggs, and all six frogs injected with 250 µg laying 6825 eggs on average (Fig. 2B). None of the six females injected with 200 µg of pLH nor the six injected with 250 µg of it laid any eggs (Fig. 2B). When accounting for the body mass of the females injected, on average all the three hormones were

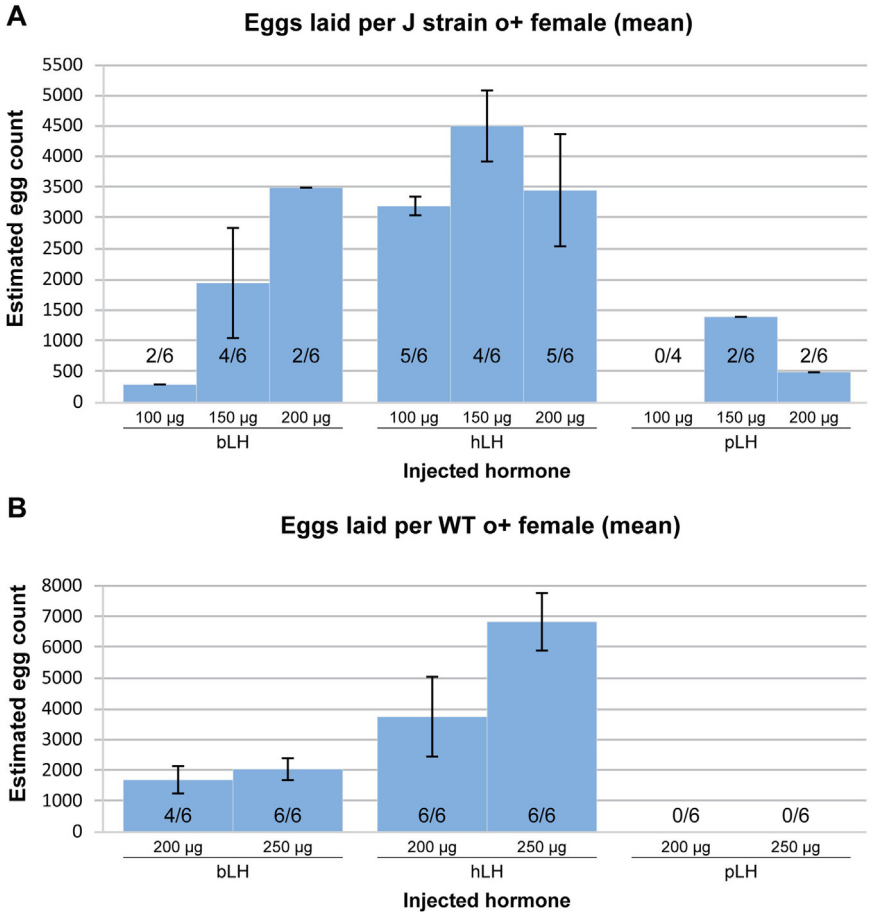

Fig. 2. Efficiency of spawning in J strain and WT o+ females boosted with either bLH, hLH, or pLH. (A) J strain o+, and (B) WT o+ *X. laevis* females were boosted with either bLH, hLH, or pLH. The height of each bar indicates the average number of eggs laid per female with the error bars showing the standard error of the mean. The ratios within or above each bar represent the corresponding number of females that laid eggs per number of females boosted. Only females that laid were included in the calculations of average number of eggs laid and the standard error of the mean. The amount and type of hormone used are indicated beneath the bars.

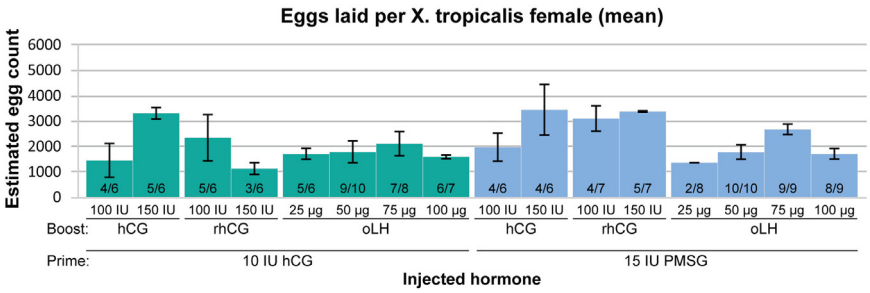

Fig. 3. Spawning efficiency in *X. tropicalis* females first primed with either hCG or PMSG and then boosted with hCG, rhCG, or oLH. *X. tropicalis* females were primed with either 10 IU of hCG (green bars) or 15 IU of PMSG (blue bars). The height of each bar indicates the average number of eggs laid per female with the error bars showing the standard error of the mean. The ratios within each bar represent the number of females that laid eggs per number of females boosted. Only females that laid were included in the calculations of average number of eggs laid and the standard error of the mean. The amount and type of hormone used are indicated on the bottom of the graph.

tested at concentrations spanning a comparable range to oLH (Table 1). In conclusion, only human LH, and not bLH nor pLH, was as effective as ovine LH at inducing egg laying in *X. laevis*.

3.3. Boosting X. tropicalis females with oLH promotes egg laying

X. tropicalis is the other species within the *Xenopus* genus that is commonly used as a developmental biology model system. As a final test, we decided to investigate whether, just as in *X. laevis*, boosting with oLH could be used to promote egg laying in *X. tropicalis*. Furthermore, we also investigated whether egg laying response to the boosting hormone tested, was differentially affected by prior priming of the females with either 10 IU of hCG or 15 IU of PMSG.

Of the individuals primed with hCG and then boosted with 100 IU of hCG four in six produced an average of 731 eggs, and five in six boosted with 150 IU produced an average of 1651 eggs (Fig. 3). Surprisingly, the females gave a mixed response to recombinant hCG (rhCG). Five of the six females boosted with 100 IU rhCG produced an average of 1174 eggs but only three of the six boosted with 150 IU laid 573 eggs (Fig. 3). The females responded more consistently to oLH than they did to rhCG, though the average clutch size did not reach the amount resulting from boosting with 150 IU of hCG. Five of six females boosted with 25 µg of oLH produced an average of 860 eggs, nine of ten boosted with 50 µg produced 898, seven of eight boosted with 75 µg produced 1057, and six of seven boosted with 100 µg produced 803 (Fig. 3).

The main observable difference between priming with 10 IU of hCG and 15 IU of PMSG was the much better response to boosting with rhCG in the PMSG primed females. Among those, four of six boosted with 100 IU of hCG produced an average of 989 eggs, and four of six boosted with 150 IU laid an average of 1720 eggs (Fig. 3). Among the females boosted with rhCG four of seven boosted with 100 IU produced an average of 1548 eggs and five of seven boosted with 150 IU produced an average of 1686 eggs (Fig. 3). PMSG priming did not seem to have as much effect on increasing the clutch sizes of animals boosted with oLH; nonetheless, these females still laid eggs in numbers sufficient for experimental work. Two of eight individuals boosted with 25 µg produced an average of 688 eggs, all ten boosted with 50 µg produced 894, all nine boosted with 75 µg produced 1338, and eight of the nine boosted with 100 µg produced 860 (Fig. 3). In the *X. tropicalis* oLH trials we tested a range of concentrations from 1.56 µg to 6.45 µg/g body mass, with approximately 5 µg per gram providing the largest batch size (Table 1). In conclusion, as in *X. laevis,* boosting with oLH promotes efficient egg laying in *X. tropicalis* at clutch sizes sufficiently large for experimental work. Furthermore, priming with 15 IU of PMSG produces essentially similar response to the boosting hormone as priming with 10 IU of hCG.

4. Discussion

In this paper, we describe a convenient and efficient method for induction of ovulation in *Xenopus* that can be used in lieu of the established protocol relying on injection of hCG into the dorsal lymph sac (Sive et al., 2000). The only difference is substitution of hCG with oLH, with the overall technique still based on dorsal lymph sac injection and thus not requiring any additional training. Our results clearly demonstrate that in both WT and J strain *X. laevis* females, oLH is capable of inducing ovulation as efficiently as hCG independent of prior breeding history. The numbers of eggs laid are generally comparable between the two hormones with the only potential exception being that WT virgin females do not appear to lay as many eggs when injected with oLH. It is possible that a higher amount if oLH may increase the number of eggs laid, but in general we found that approximately 2.5 µg/g of oLH was sufficient to replicate results produced by hCG.

oLH is also effective at inducing ovulation in *X. tropicalis*. We tested a range of concentrations from approximately 1.5 µg to 6.5 µg per gram of body mass. We observed that the females respond best to 5 µg of oLH per gram of body mass. At this concentration they spawned at frequencies surpassing those of females injected with hCG (94% vs 75%), however oLH boosted females never produced as many eggs as those boosted with hCG. In this case, increasing the amount of oLH may not be a viable approach for increasing the number of eggs laid, as in our experiments higher concentrations actually decreased the average number of eggs laid. Finally, we observed that *X. tropicalis* primed with either hCG or PMSG responded equally well to boosting with oLH.

In *X. laevis* we also tested responses to three additional Luteinizing Hormones, bovine, human and porcine LH. Only hLH consistently produced results, which suggests it may also serve as a good substitute for boosting with hCG. Although we were disappointed that not all of the mammalian hormones tested produced a similarly high ovulation response, this observation is not at all surprising. A previous comparison of LH preparations from nine species of eutherian mammals demonstrated a broad range in their ability to promote *X. laevis* oocyte maturation in vitro, with the ovine derived LH being the most effective (Licht and Papkoff, 1976).

In conclusion, we found that boosting *Xenopus* with oLH can be used in place of hCG to induce ovulation, at a dose of approximately 2.5 µg/g oLH in *X. laevis* and twice that concentration in *X. tropicalis*. Furthermore, as a practical comparison, the oLH available from the National Hormone and Peptide Program is priced at $150.00 per 10 mg, which ends up costing $3.00 per boosting injection per WT *X. laevis* female. The hCG available from Sigma-Aldrich (Catalog # CG10) when bought in bulk of 10 vials each containing 10,000 IU is priced at $956.00 which brings the cost of

each boosting injection to $4.78. Thus, the use of oLH will not only aid in avoiding any disruption in experimental work resulting from shortages in commercially available hCG but might also aid in lowering the overall experimental costs.

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References

Bergers, A.C., Li, C.H., 1960. Amphibian ovulation in vitro induced by mammalian pituitary hormones and progesterone. Endocrinology 66, 255–259. http://dx.doi.org/10.1210/endo-66-2-255.

Boorstein, W.R., Vamvakopoulos, N.C., Fiddes, J.C., 1982. Human chorionic gonadotropin β-subunit is encoded by at least eight genes arranged in tandem and inverted pairs. Nature 300, 419–422. http://dx.doi.org/10.1038/300419a0.

Choi, J., Smitz, J., 2014. Luteinizing hormone and human chorionic gonadotropin: origins of difference. Mol. Cell Endocrinol. 383, 203–213. http://dx.doi.org/10.1016/j.mce.2013.12.009.

Elkan, E.R., 1938. The Xenopus pregnancy test. Br. Med. J. 2, 1253–1274.2.

Elkan, R.E., 1946. A new test for pregnancy. Postgrad. Med. J. 22, 87–93.

Gurdon, J.B., Hopwood, N., 2000. The introduction of *Xenopus laevis* into developmental biology: of empire, pregnancy testing and ribosomal genes. Int. J. Dev. Biol. 44, 43–50.

Hogben, L., Charles, E., Slome, D., 1931. Studies on the pituitary. J. Exp. Biol. 8, 345–354.

Licht, P., Papkoff, H., 1976. Species specificity in the response of an in vitro amphibian (*Xenopus laevis*) ovulation assay to mammalian luteinizing hormones. Gen. Comp. Endocrinol. 29, 552–555. http://dx.doi.org/10.1016/0016-6480(76)90039-3.

Licht, P., Licht, P., Papkoff, H., Papkoff, H., Farmer, S.W., Farmer, S.W., Muller, C.H., Muller, C.H., Tsui, H.W., Tsui, H.W., Crews, D., Crews, D., 1976. Evolution of gonadotropin structure and function. Recent Prog. Horm. Res. 33, 169–248.

Massey Jr, F.J., 1951. The Kolmogorov-Smirnov test for goodness of fit. J. Am. Stat. 46, 68–78. http://dx.doi.org/10.1080/01621459.1951.10500769.

Naylor, S.L., Chin, W.W., Goodman, H.M., Lalley, P.A., Grzeschik, K.H., Sakaguchi, A.Y., 1983. Chromosome assignment of genes encoding the alpha and beta subunits of glycoprotein hormones in man and mouse. Somat. Cell Mol. Genet. 9, 757–770.

Ogawa, A., Dake, J., Iwashina, Y.-K., Tokumoto, T., 2011. Induction of ovulation in Xenopus without hCG injection: the effect of adding steroids into the aquatic environment. Reprod. Biol. Endocrinol. 9, 11. http://dx.doi.org/10.1186/1477-7827-9-11.

Practice Committee of American Society for Reproductive Medicine, Birmingham, Alabama, 2008. Gonadotropin preparations: past, present, and future perspectives. Fertil. Steril. 90, S13–S20. http://dx.doi.org/10.1016/j.fertnstert.2008.08.031.

Session, A.M., Uno, Y., Kwon, T., Chapman, J.A., Toyoda, A., Takahashi, S., Fukui, A., Hikosaka, A., Suzuki, A., Kondo, M., van Heeringen, S.J., Quigley, I., Heinz, S., Ogino, H., Ochi, H., Hellsten, U., Lyons, J.B., Simakov, O., Putnam, N., Stites, J., Kuroki, Y., Tanaka, T., Michiue, T., Watanabe, M., Bogdanovic, O., Lister, R., Georgiou, G., Paranjpe, S.S., van Kruijsbergen, I., Shu, S., Carlson, J., Kinoshita, T., Ohta, Y., Mawaribuchi, S., Jenkins, J., Grimwood, J., Schmutz, J., Mitros, T., Mozaffari, S.V., Suzuki, Y., Haramoto, Y., Yamamoto, T.S., Takagi, C., Heald, R., Miller, K., Haudenschild, C., Kitzman, J., Nakayama, T., Izutsu, Y., Robert, J., Fortriede, J., Burns, K., Lotay, V., Karimi, K., Yasuoka, Y., Dichmann, D.S., Flajnik, M.F., Houston, D.W., Shendure, J., DuPasquier, L., Vize, P.D., Zorn, A.M., Ito, M., Marcotte, E.M., Wallingford, J.B., Ito, Y., Asashima, M., Ueno, N., Matsuda, Y., Veenstra, G.J., Fujiyama, A., Harland, R.M., Taira, M., Rokhsar, D.S., 2016. Genome evolution in the allotetraploid frog Xenopus laevis. Nature 538, 336-343.PMID:27762356 PMCID:PMC5313049.

Sive, H.L., Grainger, R.M., Harland, R.M., 2000. Early Development of *Xenopus laevis*: A Laboratory Manual.

Thornton, V.F., 1971. A bioassay for progesterone and gonadotropins based on the meiotic division of Xenopus oocytes in vitro. Gen. Comp. Endocrinol. 16, 599–605. http://dx.doi.org/10.1016/0016-6480(71)90125-0.