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—Original Article—

Characterization of Alpha-fetoprotein Levels in Three Dolphin Species: Development of Sensitive Immunoassays for Analysis of the Pregnancy-associated Variations

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Abstract. A single radial immunodiffusion (SRID) assay and a chemiluminescent immunoassay (CLIA) were initially developed for alpha-fetoprotein (AFP) of the striped dolphin. Utilizing these developed assays, we investigated pregnancy-associated changes in the levels of AFP in the sera of fetuses and pregnant females of three dolphin species; samples were either collected from captive individuals or obtained as fishery by-products. The concentrations of AFP in the fetal serum ranged from 419.0 to 2026.3 μg/ml in the striped dolphin, 12.6 to 1218.7 μg/ml (for an AFP equivalent; eqAFP) in the common bottlenose dolphin and 770.6 to 3129.1 μg eqAFP/ml in the Risso's dolphin. AFP levels decreased with increased fetal size in fetuses over 20 cm in length. The concentrations of AFP in sera of pregnant females ranged from 7.18 to 8068.7 ng/ml in the striped dolphin, 6.6 to 1241.1 ng eqAFP/ml in the common bottlenose dolphin and 3.4 to 2868.7 ng eqAFP/ml in the Risso's dolphin. The levels in most pregnant females were equal to or lower than those found in males and nonpregnant individuals, although a few pregnant females exhibited extremely high levels (in the range of hundreds to thousands of nanograms per milliliter). Such high levels of AFP were not observed during pseudopregnancy. To our knowledge, this is the first report on basal profiles for serum AFP levels in small odontocetes. The profiles indicated that AFP may play a significant role during embryonic development, although maternal levels do not appear to be a diagnostic biomarker for monitoring pregnancy.

Key words: Alpha-fetoprotein (AFP), Cetaceans, Chemiluminescent immunoassay (CLIA), Single radial immunodiffusion (SRID), Striped dolphin

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Although cetacean species are kept in captivity in a wide variety of conditions, many of the holding facilities make considerable efforts to ensure the health and reproductive fitness of the animals. In general, the programs for managing the breeding of captive cetaceans are reasonably effective, using both controlled natural breeding and advanced reproductive technologies such as artificial insemination and diagnostic ultrasound [1]. However, there is still significant room for improvement in these programs in terms of the accurate detection and continuous monitoring of pregnancy [2, 3].

Although reproductive physiology of almost all cetacean species has been largely unknown, some physiological characteristics have been reported for dolphin species. The common bottlenose dolphin (*Tursiops truncatus*) spontaneously ovulates and has estrous cycles completing in 29 to 42 days [4, 5]. However, seasonal reproductive activity varies in the common bottlenose dolphin; for example, some exhibit polyestrous, are seasonally polyestrous, and exhibit anestrous with one- to two-year intervals [4–7]. Pregnancy period and breeding season vary with dolphin species. In the striped dolphin (*Stenella coeruleoalba*), the pregnancy period was 13.2 months, and the breeding season occurred during winter and early summer [8, 9]; in the common bottlenose dolphin, the pregnancy period was 12.2 months, and breeding occurred throughout the year (especially early spring to mid-autumn) [7, 10–12]; and in the Risso's dolphin (*Grampus griseus*), the pregnancy period was 13 to 14 months, and the breeding season occurred during the summer [13].

In land mammals, a number of hormones and proteins either appear for the first time or greatly increase in the maternal circulation during pregnancy. Many of these hormones and proteins are not produced by the mother but are of fetal-placental origin. Such compounds, including chorionic gonadotropin (CG), placental lactogen (PL) and pregnancy-associated glycoprotein (PAG), have been used as markers for detecting and/or monitoring pregnancy in several mammalian species [14–17].

A previous study on pregnancy-associated proteins or hormones in cetacean species identified LH-like compounds [18] that appeared to be expressed in the placenta of the common bottlenose dolphin. Although these putative gonadotropins are possible candidates for use as markers in the detection and monitoring of pregnancy, they

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have not been exploited for this purpose to date. With respect to reproductive steroid hormones, increased progesterone levels in the plasma is an indicator of ovarian activity or ovulation in captive cetaceans [2, 19]; however, use of progesterone levels for pregnancy monitoring may be problematic because of the possibility of a false positive diagnosis due to pseudopregnancy [20, 21]. Therefore, development of accurate methods for detection and monitoring of pregnancy is required for the proper management of reproduction in captive cetaceans. In addition, in contrast to humans and domesticated mammals, our understanding of the physiological events associated with pregnancy are limited in cetacean species. Progress in this field of research would be aided by the identification of reliable pregnancy-related biomarkers in cetaceans.

Alpha-fetoprotein (AFP), a tumor-associated fetal glycoprotein, is present at relatively high concentrations in fetal and neonatal sera, as well as in the amniotic fluid of various mammalian species. It is synthesized by the fetal liver and yolk sac [22, 23]. Although the precise biological functions of this protein are unknown, a number of AFP activities have been reported such as immunosuppression, regulation of cell growth and binding to ligands such as bilirubin, fatty acids, retinoids, steroids, heavy metals, dyes, flavonoids, phytoestrogens, dioxin and various drugs [24]. In addition, AFP has been shown to bind strongly to estrogens [25, 26]. In humans, AFP levels in the maternal circulation have been used to monitor fetal condition during pregnancy: significant elevation of AFP levels is associated with fetal neural tube defects, while a decrease can indicate Down's syndrome pregnancies in the second trimester [27]. In a normal pregnancy, the levels of circulating AFP in the fetus increase, reach maximal values at approximately 13 weeks of gestation and then decrease to term. In the maternal serum, the AFP levels peak at around 32 weeks of gestation and thereafter decline until parturition [28].

To date, AFP has been used as a biomarker for monitoring a number of aspects of reproduction such as the initiation and progress of pregnancy and pathological defects in the mother or fetus. Recently, we succeeded in detecting, purifying and characterizing AFP in the serum of fetal striped dolphins; this was the first time such information had been obtained from a cetacean species. We were able to use these new data to initiate an investigation of the role of AFP in the reproductive physiology of this species [3]. As a first step, we generated and validated an AFP specific antibody that could be used in the development of a quantitative AFP assay. The aims of the present study were 1) to develop a highly sensitive chemiluminescent immunoassay (CLIA) and a conventional single radial immunodiffusion (SRID) assay for AFP and 2) to quantify the serum levels of AFP in the striped dolphin and two other species of dolphin, the common bottlenose dolphin and the Risso's dolphin. These dolphins are distributed worldwide in tropical and temperate waters and belong to members of the delphinidae family in the suborder of Odontoceti [8, 11, 13, 29]. As part of the latter analysis, special attention was paid to obtaining profiles of maternal and fetal AFP levels that could then be correlated with the progress of pregnancy or pseudopregnancy. This analysis should enable us to identify the involvement of AFP in aspects of dolphin reproductive physiology and to ascertain whether the profiles could provide a reliable pregnancy-associated biomarker.

Materials and Methods

Experimental animals and tissue samples

Striped dolphins, common bottlenose dolphins and Risso's dolphins were caught by drive fishery off the Pacific coast of Taiji, Wakayama Prefecture, Japan, from December 2007 to February 2008 and in January 2010. Blood and tissue samples were not obtained for experimentation but as fishery by-products and were collected as soon as possible after the death of animals. However, animal handling by the fishery workers was carried out according to the method of Olsen [30] for the striped dolphin (samples from 2010), the common bottlenose dolphin and the Risso's dolphin, which is the most efficient, safe and humane method without distress for animals [30, 31]. Each species included pregnant females and fetuses, nonpregnant mature females, immature females and mature males: (n=11, 11, 15, 7, 7, respectively, for the striped dolphin; n=18, 14, 7, 1, 7, respectively, for the common bottlenose dolphin, n=9, 7, 5, 5, 8, respectively, for the Risso's dolphin). Adult and fetal blood samples were taken from the abdominal cavity and allowed to stand at 4 C overnight. The samples were centrifuged (1,400 *g*, 20 min), and the sera were collected in Sepaclen tubes (Eiken Kizai, Tokyo, Japan). Serum samples were stored at –30 C until use. Fetal body length data were provided by the Fisheries Research Agency.

Serum samples were also obtained from a captive and well-trained healthy female common bottlenose dolphin kept at the Kamogawa Sea World aquarium (Chiba prefecture) to obtain information on changes in serum AFP levels over time. Serum samples were taken once or twice monthly from December 2009 to June 2010 (n=11). The trainer instructed the dolphin to present their tail fluke, and blood samples were collected from the fluke blood vessel. Then the collected blood was placed in a vacuum blood sampling tube (Venoject II Autosep, Terumo, Tokyo, Japan). After centrifugation at 1,700 *g* for 5 min at room temperature, the serum was collected and stored at –20 C until assayed.

Purification of dolphin AFP

Purified AFP was prepared from fetal serum samples as described by Morita *et al*. [3]. The protein concentrations in purified AFP samples were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) as the reference standard. Purified AFP was stored at –80 C until use.

Antisera

Rabbit antiserum against striped dolphin AFP (anti-dolphin AFP) was prepared as described previously [3].

Single radial immunodiffusion (SRID) procedure

Quantification of AFP in fetal serum was performed using SRID on a 1% agarose gel containing anti-dolphin AFP, as previously described [32]. The purified AFP (25–400 μg/ml) and fetal serum were serially diluted (see Figures and corresponding legends for dilutions) with 0.01 M phosphate buffered saline (PBS; pH7.0), and 5 μl of these diluted products were applied to the plates. The plates were incubated at room temperature for 2 days, and the diameters of the immunoprecipitin rings that formed were measured.

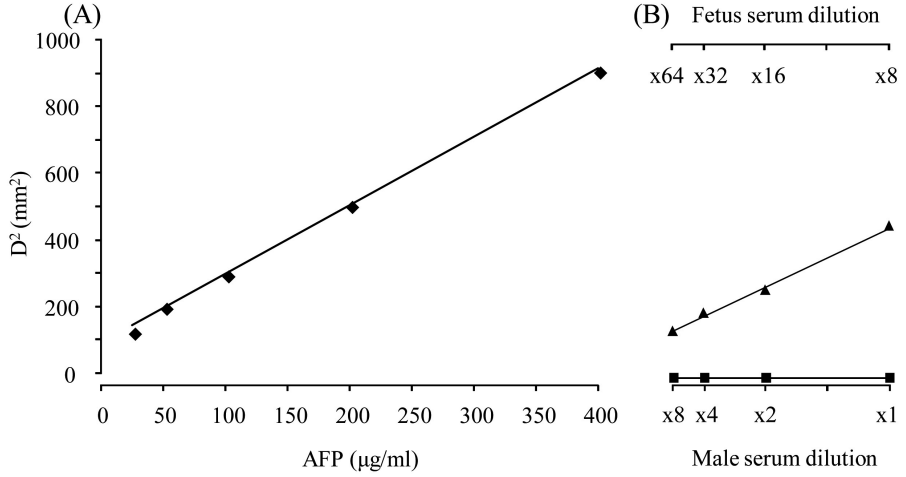

Fig. 1. Practical standard curve (A) produced using the single radial immunodiffusion (SRID) procedure for alpha-fetoprotein (AFP) of the striped dolphin and the corresponding dilution curves (B) for sera from fetuses (closed triangle) and males (closed square). D: diameter (D) of precipitin rings.

Preparation of IgG and acridinium–labeled F(ab')$_2$

Immunoglobulin G (IgG) was purified from rabbit anti-dolphin AFP by anion exchange chromatography using DE52 (Whatman International, Maidstone, Kent, UK) as previously described [33]. F(ab')$_2$ was sequentially prepared by a peptic digestion of the IgG according to the method of Kato *et al.* [34]. The digests were applied to a Superdex 200 (GE Healthcare UK, Buckinghamshire, UK) gel filtration column (1 cm × 31 cm) fitted to an FPLC system (GE Healthcare UK) to separate F(ab')$_2$ and Fc' fragments. The prepared F(ab')$_2$ was labeled with acridinium, 4-(2-succinimidyloxycarbonyl-ethyl) phenyl-10-methylacridinium-9-carboxylate (Dojindo Chemical, Kumamoto, Japan) according to the manufacturer's instructions. Excess acridinium was removed by dialysis against 0.1 M PBS (pH 6.3) overnight at 4 C.

Chemiluminescent immunoassay (CLIA) procedure

The CLIA was carried out usingthe method of Fukada *et al.* [35] unless otherwise stated. The assay was performed using 96-well plates (LIA-Plate; Greiner, Frickenhausen, Germany) in wells that had been coated with an anti-AFP IgG solution (5–40 μg/ml anti-dolphin AFP IgG in 0.01 M PBS, pH 7.0; 150 μl/well) by incubation for 4 h at room temperature. After washing and blocking the plates, serum samples or standards (i.e., purified striped dolphin AFP), which had been diluted as appropriate with PBS containing 1% BSA and 0.1% NaN_3 (PBS-BSA), were added at a volume of 100 μl per well and incubated for 2 h at room temperature. After washing the plates as described above, 100 μl of the acridinium-labeled anti-dolphin AFP F(ab')$_2$ (diluted at 1:100,000–800,000 in the PBS-BSA) was added to each well and incubated for 2 h at room temperature. The plates were then washed, and the amount of bound antibody was determined by measuring luminescence in a Luminescencer-JNR (ATTO, Tokyo, Japan). All assays were carried out in duplicate or triplicate.

Assay validation

Parallelism between standard and sample regression curves was tested by an analysis of covariance [36] with the significance level set at $P>0.05$.

Measurement of progesterone

Serum progesterone was measured by competitive EIA using a progesterone enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA) in accordance with the manufacturer's instructions.

Results

Development of SRID

The standard curve from the SRID assay of striped dolphin AFP is shown in Fig. 1. Serial dilutions of purified AFP formed standard precipitin rings that produced a practical standard curve ($R^2>0.999$) in the range of 25 to 400 μg/ml. The serial dilutions of fetal serum showed sufficient parallelism to the standard curve, while no immunoreaction was observed in the male serum.

Development of CLIA

Tests were performed to identify the optimal dilution of IgG for the coating step (Fig. 2A) and for the acridinium-labeled F(ab')$_2$ antibody (Fig. 2B). Comparison of wells coated with IgG in the range 5 to 40 μg/ml showed that the maximum relative light unit (RLU) with 5 μg/ml IgG appeared to be lower than the maximum RLUs of other concentrations. Since the pattern of the standard curve was not altered by decreasing the coating concentration from 40 to 10 μg/ml, we chose the latter coating as the condition for the assays. Subsequently, the optimal dilution of labeled F(ab')$_2$ antibody was tested using four different dilutions (1: 100,000, 1: 200,000, 1: 400,000 and 1: 800,000). The resulting RLUs decreased with increased dilution of the antibody; the optimal RLU for the Luminescencer-JNR detection unit (the maximum RLU at 10,000–20,000) was achieved at the 1:100,000 dilution. Therefore, this dilution was used for the CLIA.

The practical standard curve for the CLIA of dolphin AFP is shown in Fig. 3A. A linear standard line with an adequate coefficient

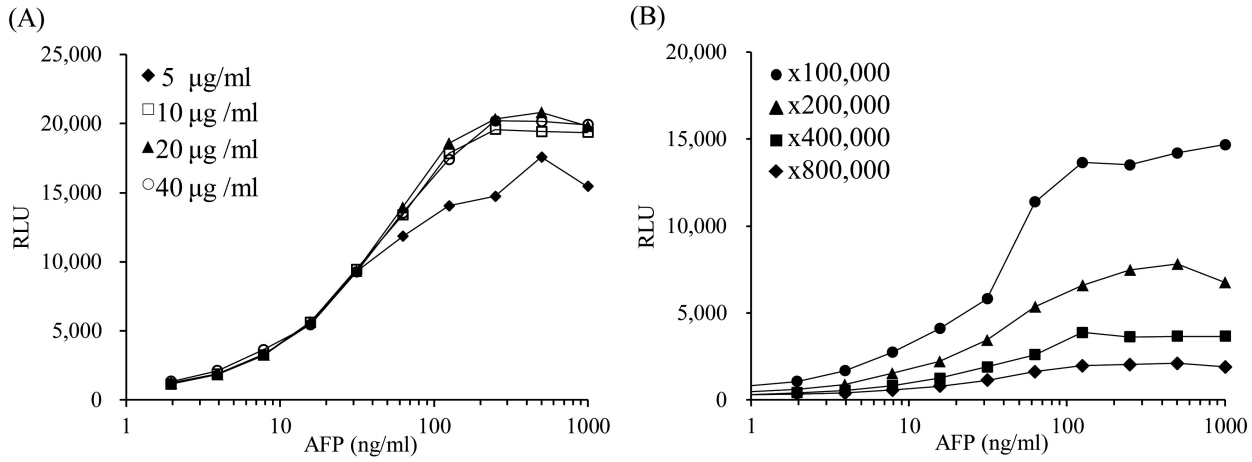

Fig. 2. Optimization of the chemiluminescent immunoassay for alpha-fetoprotein (AFP) of the striped dolphin using different concentrations of the coating IgG antibody (A) and dilutions of acridinium-labeled F (ab')$_2$ antibody (B). RLU: relative light unit.

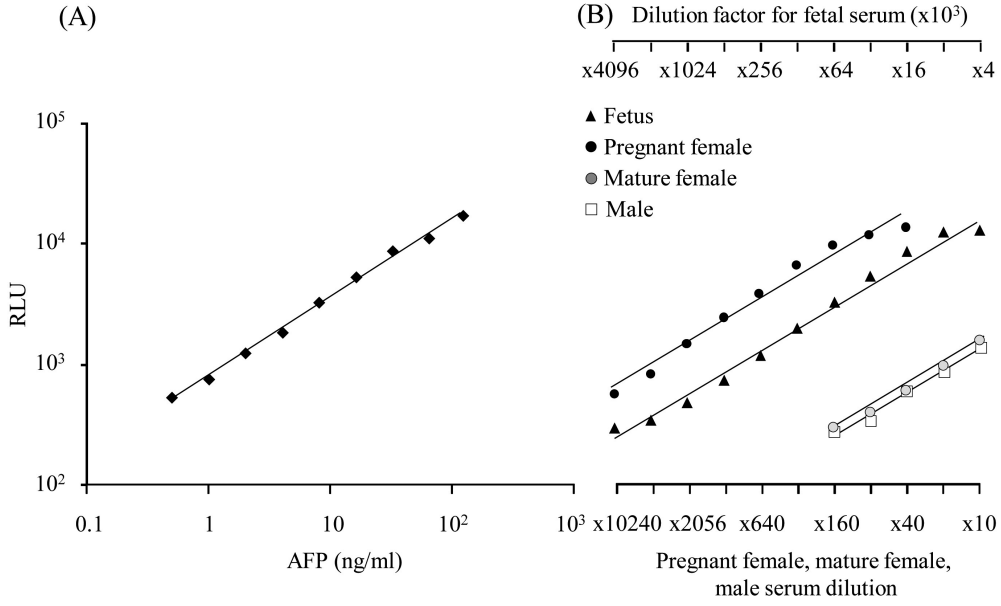

Fig. 3. Practical standard curve (A) of the chemiluminescent immunoassay (CLIA) for the alpha-fetoprotein (AFP) of the striped dolphin and the corresponding dilution curves (B) for fetal serum (closed triangle), pregnant female serum (closed circle), mature female serum (gray circle) and male serum (open square).

of determination (R^2>0.995) was obtained across the range 0.49 to 125 ng/ml.

The serial dilutions of fetal and pregnant female serum revealed sufficient parallelisms (P>0.05) with the standard curve of AFP (Fig. 3B). Such parallelisms were also obtained between the standard curve and serial dilutions of sera of nonpregnant males and females, although they were observed in a limited range with the low dilution factors (10 to 160 times dilution).

Cross-reactivity with sera of fetuses and pregnant females in other small odontocetes

Serum samples obtained from fetal and pregnant female common bottlenose dolphins and Risso's dolphins produced dilution curves that paralleled the AFP standards in both the SRID assay (Fig. 4) and the CLIA (Fig. 5), indicating that these AFP assays can be applied to determine the relative levels of AFP in these species.

Precision and recovery tests of the assay

Precision tests were performed using various concentrations of purified AFP. Inter- and intra-assay coefficients of variation are

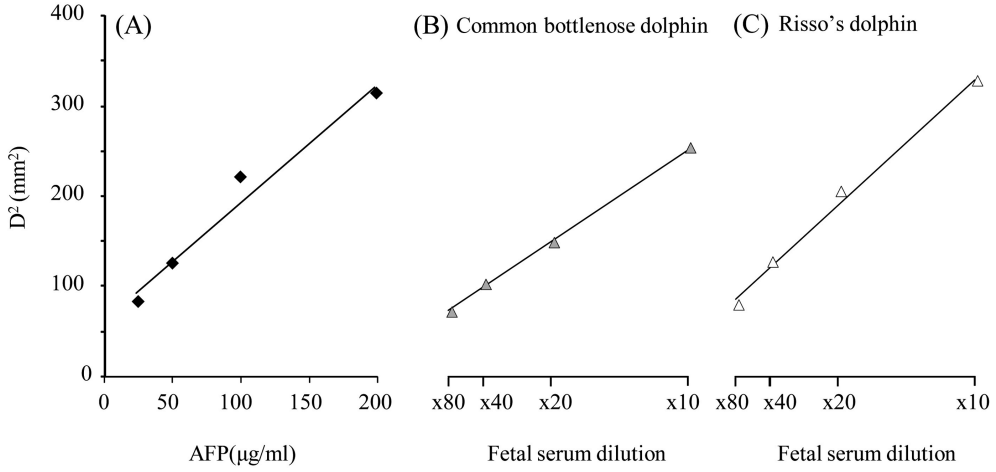

Fig. 4. Immuno-cross-reactivities of alpha-fetoprotein (AFP)-positive reactions in the single radial immunodiffusion (SRID) assay. Serial dilutions of purified AFP (A), as well as serial dilutions of sera from the common bottlenose dolphin (B) and the Risso's dolphin (C), were applied to the SRID plate. D: diameter (D) of precipitin rings.

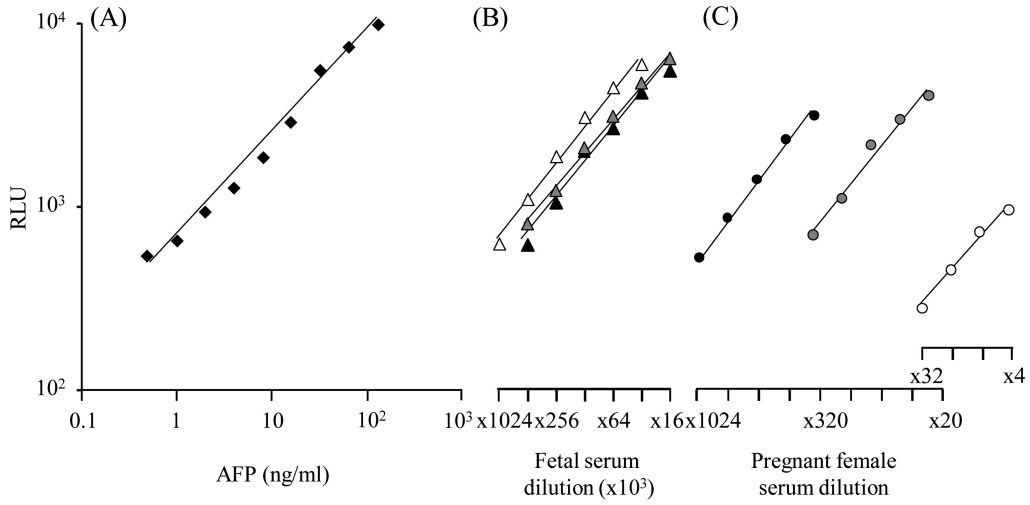

Fig. 5. Immuno-cross-reactivities of alpha-fetoprotein (AFP) in the chemiluminescent immunoassay (CLIA). Serial dilutions of purified AFP (A), as well as serial dilutions of fetal (B) and pregnant female sera (C) from the striped dolphin (closed triangle and closed circle, respectively), the common bottlenose dolphin (gray triangle and gray circle, respectively) and the Risso's dolphin (open triangle and open circle, respectively), were applied to sample wells. RLU: relative light unit.

shown in Table 1. The inter-assay coefficient of variation ranged from 0.98% to 4.10%, while the intra-assay coefficient variation ranged from 1.55% to 4.07%. In order to evaluate the assay recovery rate, various concentrations of purified AFP (0.195, 0.39, 0.78, 1.56, 3.125 and 6.25 ng) were added to male serum. Quantification of AFP in these supplemented serum samples yielded recovery rates in the range of 96.06% to 104.51% (Table 2).

Measurement of AFP levels in fetal and pregnant female sera

The relationship between AFP level in fetal serum and fetal length was analyzed in three dolphin species (Fig. 6A, B, C). AFP concentrations in fetal serum ranged from 419.0 to 2026.3 μg/ml for the striped dolphin, 12.6 to 1218.7 μg/ml (for a striped dolphin AFP equivalent; eqAFP) for the common bottlenose dolphin and 770.6 to 3129.1 μg eqAFP/ml for the Risso's dolphin. Fetal AFP levels decreased with increased size in fetuses over 20 cm in length. Negative correlations were evident for the striped dolphin ($R=-0.7$, $P<0.05$) and the common bottlenose dolphin ($R=-0.87$, $P<0.05$); although the Risso's dolphin showed a similar trend, the correlation was not significant ($R=-0.61$, $P<0.05$).

Next, we compared AFP levels in the sera of pregnant females in the three species and looked to see if these levels varied during

Table 1. Precision tests of the chemiluminescent immunoassay for alpha-fetoprotein (AFP)

	AFP concentration (ng/ml)	N*	CV** (%)
Intra-assay variations	34.11 ± 0.9	5	1.23
	17.87 ± 0.97	5	2.45
	7.94 ± 0.17	5	0.98
	4.00 ± 0.37	5	4.10
	1.95 ± 0.13	5	3.18
Inter-assay variations	36.62 ± 4.24	5	4.07
	17.02 ± 1.47	5	3.88
	8.19 ± 0.51	5	2.77
	3.88 ± 0.13	5	1.55
	1.82 ± 0.12	5	2.92

Values for AFP concentrations are expressed as means ± standard deviation. *Number of tests. **Coefficient of variation.

Table 2. Recovery tests of the chemiluminescent immunoassay for alpha-fetoprotein (AFP)

AFP concentration		N*	Recovery (%)
Added AFP (ng)	Mean (ng/well)		
0	0.66	5	–
0.195	0.86	5	100.28
0.39	1.05	5	99.66
0.78	1.41	5	98.67
1.56	2.32	5	104.51
3.125	3.84	5	101.46
6.25	6.67	5	96.06

*Number of tests.

pregnancy. Additionally, we compared the AFP levels of pregnant females to those of nonpregnant females, immature females and mature males (Fig. 6a, b, c). Serum AFP concentrations in pregnant females ranged from 7.18 to 8068.7 ng/ml for the striped dolphin, 6.6 to 1241.1 ng eqAFP/ml for the common bottlenose dolphin and 3.4 to 2868.7 ng eqAFP/ml for the Risso's dolphin. Thus, in all three species, extremely high levels of AFP were observed in the serum of some pregnant females. To determine whether this variation was associated with stages of pregnancy, we examined AFP levels in relation to fetal lengths. We could not find any clear relationship, although there was a tendency for higher levels of AFP in the middle stages of pregnancy. The average concentrations of serum AFP in mature nonpregnant females, immature females and mature males were low: 14.2 ± 9.1 (n=15), 12.2 ± 10.6 (n=7) and 11.0 ± 6.1 (n=7) ng/ml, respectively, for the striped dolphin; 12.7 ± 5.2 (n=6, excluding data of one individual exhibiting an abnormally high level of AFP), 8.7 (n=1) and 6.6 ± 2.5 (n=6) ng/ml, respectively, for the common bottlenose dolphin; and 7.2 ± 4.8 (n=5), 1.9 ± 1.6 (n=5) and 8.3 ± 5.5 (n=8) ng/ml, respectively, for the Risso's dolphin. These average levels were 8.5 to over 300 times lower than those of pregnant females (1370.59 ± 2444.14 for the striped dolphin, 107.57 ± 279.68 for the common bottlenose dolphin, 491.29 ± 923.07 for the Risso's dolphin). However, some pregnant females (n=4 for the striped dolphin, 12 for the common bottlenose dolphin and 5 for the Risso's dolphin) exhibited AFP levels in the same range as those of nonpregnant and immature females and of males.

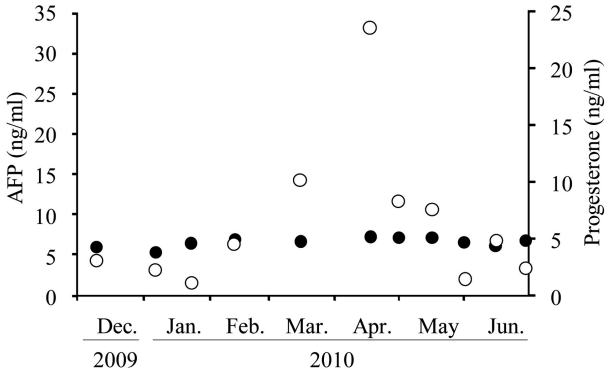

Fig. 7. Changes in serum levels of alpha-fetoprotein (AFP, closed circles) and progesterone (open circles) in a captive female common bottlenose dolphin during a period of possible pseudopregnancy.

Measurement of serum AFP levels in a captive female common bottlenose dolphin

The changes in serum AFP and progesterone concentrations in a captive common bottlenose dolphin are shown in Fig. 7. Serum progesterone levels rose during the initial assessment period to a maximum level of 23.59 ng/ml and then declined thereafter. The dolphin was not pregnant during this period but did display mating behavior. In contrast to the progesterone levels, serum AFP levels ranged from 5.34 to 7.26 ng eqAFP/ml with little variation during the period of assessment.

Discussion

In humans, AFP has been shown to be a practical biomarker of the initiation and progress of pregnancy and for monitoring aspects of maternal and fetal health, including pathological defects [28]. As it is desirable to have a reliable biomarker for achieving efficient management of reproductive fitness in captive cetaceans and for investigating these reproductive mechanisms, we initiated an investigation using the AFP protein and transcript in the striped dolphin [3]. In our earlier study, a sufficiently large amount of purified AFP of the required quality was obtained to enable development of a specific anti-AFP antibody; we also identified pregnancy-associated changes in AFP mRNA expression in the synthetic organ (liver) of the fetus. These results stimulated the analyses described in the present study.

Several immunoassays, such as radioimmunoassay and enzyme-linked immunoassay, have been developed for measuring AFP levels in mammals [37–44]. Since the levels of AFP in the fetus and pregnant female might be expected to vary considerably during pregnancy, we sought to develop immunoassays for AFP with two different properties: a CLIA that has high sensitivity and an SRID assay that is easy to perform. We have previously used this combination of

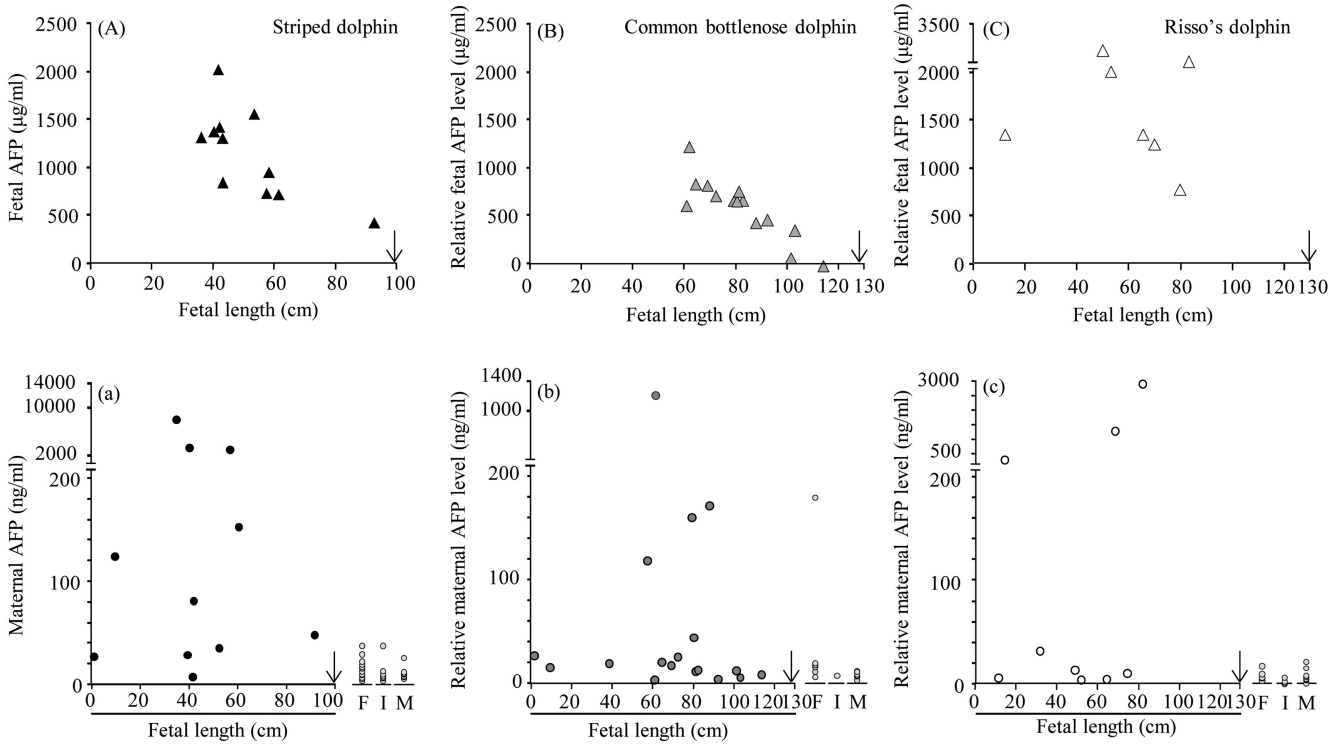

Fig. 6. Serum alpha-fetoprotein (AFP) levels in fetuses with different body lengths (panels A, B and C; triangles), in the corresponding maternal dolphins (panels a, b and c, circles), in nonpregnant mature females (F), in immature females (I) and in mature males (M) of the striped dolphin (panels Aa), the common bottlenose dolphin (panels Bb) and the Risso's dolphin (panels Cc). The typical birth length for each dolphin species [9, 46, 47] is indicated by an arrow in each graph.

methods to quantify the female-specific serum protein vitellogenin (a precursor of egg yolk in oviparous animals), which exhibits large variations in the serum (from ng/ml to mg/ml) during different phases of reproduction in fish. This methodological approach was chosen in order to reduce labor by initially performing the primary screening of serum samples with the SRID assay prior to application of the CLIA [45].

The SRID assay developed for use in dolphins appeared to provide specificity to the target antigen with no cross-reactivity to any other protein components in the serum of the striped dolphin. Thus, this protocol provided a simple procedure to quantify AFP at relatively high levels, such as can occur in fetal serum. Simultaneously, a specific and sensitive AFP CLIA was developed in the present study using an AFP antibody directly labeled with an acridinium ester. The AFP CLIA provided an efficient assay across the normal range of the protein in the sera of dolphins of both genders.

Both assays showed cross-reactions with sera of other cetacean species. The parallelisms between the standard curve and the serial dilution curves generated with sera of two other cetacean species, the common bottlenose dolphin and the Risso's dolphin, were sufficient to enable quantification of relative AFP levels in these species.

The levels of AFP were higher in the fetal serum than in the maternal circulation in all three cetacean species examined here. This effect can be explained by the fact that the major organ synthesizing dolphin AFP is the fetal liver and not the maternal placenta [3]. Based on data that indicate the typical fetal body lengths at birth are approximately 99.8 cm in the striped dolphin [9], 128 cm in the common bottlenose dolphin [46] and 130 cm in the Risso's dolphin [47], the pregnant females examined in this study are estimated to have been mainly in mid- to late-stage gestation, with a few exceptions. From this survey of a limited range of gestational ages, we conclude that the data support our previous suggestion of a tendency toward a negative correlation between circulating fetal AFP levels and fetal growth as described in our preliminary observations on expression profiles of AFP mRNA [3].

The AFP profiles in fetal sera differ among species; the physiological significance of this variation is unknown. In rats [38] and rabbits [48], which have relatively short gestation periods, AFP levels in the fetal serum peaked near term. In contrast, in sheep [49], humans [50] and cattle [51], which have relatively prolonged pregnancies, fetal AFP levels peaked in early gestation. The profiles of serum AFP levels in dolphins appeared to belong to the latter pattern. This is consistent with the fact that most cetacean species exhibit relatively long gestation periods, which lasts for a year in most mysticetes [52] and varies from 10 to 16 months in small odontocetes [53].

Like other mammals, AFP levels were kept in the ng/ml range except in a few pregnant individuals. In dolphins, variation in AFP levels did not seem to be correlated with fetal length (an indirect measure of gestational age), although some animals exhibited a tendency toward higher AFP levels around the middle stages of

pregnancy. With regard to AFP levels in the maternal circulation, several patterns have been reported depending on the species. For example, bovine maternal plasma levels remain very low throughout gestation [51]. By contrast, sheep maternal serum AFP levels are not elevated in the first two-thirds of pregnancy but instead show a tendency to rise only during the last third of pregnancy [49]. In pigs, AFP levels in the maternal serum do not rise significantly during gestation, but at mid pregnancy, some pigs have comparatively high levels of AFP and average double the levels of nonpregnant animals [39]. In rats [38] and humans [54], circulating AFP levels increase significantly during pregnancy.

The reason for these different patterns of AFP fluctuation between species is unclear. It is possible that they reflect differences in their respective placental structures. Morphologically, placentas fall into four groups according to the number of layers interposed between the maternal and fetal circulations: epitheliochorial, synepitheliochorial, endotheliochorial and hemochorial [55]. Cows and sheep have synepitheliochorial placentas, which have more layers of tissue separating the fetal and maternal blood than those of the hemochorial placentas of rats and humans [56, 57]. Histological analysis of the mature cetacean placenta shows that it falls into the epitheliochorial category, like the pig [58].

Collectively, the profiles of maternal AFP levels in dolphins most closely resembled those of pigs, perhaps because of their similar placental structures. We also identified a few individuals with exceptionally high AFP levels. Whether such high levels are normal remains to be elucidated; however, they possibly reflect pregnancy status rather than a physiological and/or pathological condition, as similarly high levels were never observed in males, nonpregnant females or pseudopregnant females. It is also possible that there could be interindividual variation in the timing of the elevation during normal gestation. The timing of the transfer of fetal AFP to the maternal circulation appeared to be under some form of regulation, as no clear relationship between AFP levels in fetal and pregnant female sera was evident ($R=0.24$, $P>0.05$, data not shown). A potential explanation for the AFP variation in maternal sera could be the presence of a transfer process of fetal AFP via a receptor-mediated mechanism in the placenta [59]; the timing of the elevation in AFP levels might be dependent on changes in the properties of the placental AFP receptor during pregnancy.

In this study, we detected low levels of AFP in the sera of non-pregnant and immature females, as well as in mature males (Fig. 6a, b, c). The presence of low levels of AFP in nonpregnant adult sera of other mammals has also been reported [60–62]. Therefore, we conclude that the low levels of AFP detected here in these groups of dolphins using the highly sensitive CLIA are clearly unrelated to pregnancy-specific activities. One nonpregnant common bottlenose dolphin female exhibited a substantially higher level of AFP. This female had a hypertrophic endometrium and may therefore have been in a post-parturition condition, or alternatively have had an abnormal reproductive or pathological status; because of this uncertainty, this animal should be regarded as an exceptional case. There is evidence from humans that high levels of AFP occur in the sera of nonpregnant women with hepatocellular carcinoma and germ cell tumors [63]. This suggests that a similar effect on AFP levels may occur in cetaceans in response to some abnormal physiological or pathological conditions.

The use of AFP profiles as a practical biomarker of a range of pregnancy-related conditions in cetacean species, such as the initiation of pregnancy, the progress of embryonic development and the detection of fetal and maternal disorder, requires a detailed knowledge and understanding of the roles of AFP. Here, we paid special attention to the following questions: 1) is the AFP level in serum of pregnant individuals always higher than in nonpregnant individuals, and 2) is there any correlation between the AFP level in serum of pregnant individuals and fetal length (i.e., embryonic development)? The first point is vitally important, as it determines whether AFP levels can diagnose pregnancy in cetacean species. Our results indicated that AFP levels were on average higher in pregnant females than nonpregnant animals, but this was not always the case for every pregnant individual. Therefore, we tentatively set the threshold for possible pregnancy at a level greater than the maximum AFP level obtained for nonpregnant individuals: 37.17 ng/ml for the striped dolphin, 19.53 ng eqAFP/ml for the common bottlenose dolphin and 16.00 ng eqAFP/ml for the Risso's dolphin. Females who exceed the threshold level may be considered as "potentially pregnant"; however, it was not possible to say whether females with levels lower than the threshold are pregnant or not. The threshold may also distinguish pseudopregnancy from true pregnancy. In the captive dolphin examined in the present study, high progesterone levels were observed for several months when this individual appeared to be in a state of pseudopregnancy, which can last for 5–6 months in the common bottlenose dolphin [20]. As this animal has been maintained with a male, early fetal loss might have occurred. However, we could not confirm the discharge of conception products including an aborted fetus following a decrease in progesterone levels; based on this situation, we concluded that this animal was in a state of pseudopregnancy. In this condition, the levels of AFP do not exhibit any correlation with progesterone levels, suggesting that the rise in AFP only occurs during "true" pregnancy. Therefore, the use of a supportive diagnosis with AFP is suggested as a means of improving the accuracy of current diagnostic tests that use progesterone to screen for pregnancy and pseudopregnancy in cetaceans.

Providing an answer to the second question was required to determine whether testing for AFP levels would provide data relevant to determining that pregnancy and embryo development were progressing normally. Unfortunately, there seemed to be no correlation between maternal AFP levels and embryonic growth, although there was some indication of a negative correlation between fetal AFP levels and embryonic growth. Overall, the investigation showed that fetal AFP levels might be a potential marker for monitoring the progress of pregnancy in cetaceans. However, practical use of this would require technical improvement in the collection of blood samples from fetuses (or alternatively from amniotic fluid).

The present report describes the development of sensitive and simple immunoassays for the quantification of AFP in the sera of small odontocetes. Using these assays, we obtained the first data on the basal profiles of serum AFP levels in pregnant and nonpregnant cetaceans and, at the same time, also in their fetuses. These profiles indicated that AFP possibly plays a significant role in embryonic development. Maternal AFP profiles did not provide a robust and

reliable biomarker of the initiation and progress of pregnancy, although they could be used for reference. By contrast, fetal AFP profiles did have the potential for application as a pregnancy-associated marker for the management of reproduction of cetaceans. The knowledge obtained in this study should increase our understanding of some of the molecular mechanisms involved in the maintenance of pregnancy in cetaceans, which will in turn allow further improvement in current captive breeding programs.

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