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Examination of oestrus-dependent alterations of bovine cervico-vaginal mucus glycosylation for potential as optimum fertilisation indicators

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Abstract

Oestrus is the period in the sexual cycle of female mammals during which ovulation occurs, where they become most receptive to mating and are most fertile. Efficient detection of oestrus is a key component in successful reproductive livestock management programmes and a major factor in the efficiency and profitability of dairy farms. Oestrus detection in cattle is most often performed by visual observation of the primary signs of oestrus, such as mounting behaviour and standing heat, to facilitate more successful prediction of optimal time points for artificial insemination. This is a time-consuming method and requires a skilled, diligent observer. Biological measurements using easily accessible biomolecules in the cervico-vaginal mucus could provide an alternative strategy to physical methods of oestrus detection, which would provide an inexpensive means of rapidly and accurately assessing the onset of oestrus. In this study, glycosylation changes in cervico-vaginal mucus from three heifers following oestrus induction were investigated as a proof of concept to assess whether potential glycosylation-based trends could be useful for oestrus stage indication. Mucus collected at different time points following oestrus induction was immobilised in a microarray format and their glycosylation interrogated with a panel of fluorescently labelled lectins, carbohydrate-binding proteins with different specificities. Individual animal-specific glycosylation patterns were observed, however each pattern followed a similar trend around oestrus. This unique oestrus-associated glycosylation was identified by a combination of relative binding of the lectins SNA-I and WFA for each animal. This alteration in cervico-vaginal mucus glycosylation could potentially be exploited in future to more accurately identify optimal fertilisation intervention points compared to visual signs. More effective oestrus biomarkers will lead to more successful livestock reproductive programmes, decreasing costs and animal stress.

Keywords

Bovine; Glycosylation; Mucus; Oestrus detection

List of abbreviations

Min, minute; h, hour; Ig, immunoglobulin; TRITC, tetramethylrhodamine isothiocyanate; CIDR, controlled internal drug release device; d, day; GuHCl, guanidine hydrochloride; HCl, hydrochloride; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline with 0.05% Tween-20; TBS, Tris-buffered saline; TBS-T, Tris-buffered saline with 0.05% Tween-20; RFU, relative fluorescence units; ; NHS, *N*-hydroxylsuccinimide; Fuc, fucose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; MS, mass spectrometry; Man, mannose.

1. Introduction

Oestrus, or heat, is the period in the sexual cycle of female mammals, excluding higher primates, during which ovulation occurs and they become most receptive to mating and are most fertile. Efficient detection of oestrus is a key component in livestock fertility and successful reproductive management programmes and a major factor in the efficiency and profitability of dairy farms (1-3). Behavioural observation and physical evidence such as mounting behaviour and standing heat are the primary external signs of oestrus (4-6). Additional behavioural signs of oestrus or secondary signs can be classified into categories such as sexual attractivity, proceptivity, and receptivity (6, 7). Oestrus detection based on the visual observation of a combination of these traits has a positive association with fertility and is the most frequently used method of determining fertilisation time at present. However efficient detection by observation is time-consuming and requires a skilled, diligent observer for 20 min five times a day (1). The short window of oestrus, typically 8 to 30 h, also complicates the task. Not all dairy cows show standing activity or definitive signs of oestrus, which also complicates the process of visual detection of oestrus, and there is also a large variation in the expression of behavioural signs between animals (5, 6, 8-10). Thus ineffective heat detection is a drawback in reproduction performance, as undetected and falsely detected oestrus is strongly associated with missed and untimely inseminations, long calving intervals, heifer replacement and reduction in potential milk and calf production, resulting in significant economic losses. These losses are compounded by a dramatic increase in infertility and reproductive disorders in dairy cattle, associated with environmental and physiological influences within dairy cattle over the past decade, and increase the need for more efficient oestrus detection (11).

Advances in technology have led to the development of a number of mechanical and automated devices to detect reproductive cycle status including tail paint, mechanical heatmount detectors

such as Kamar Heatmount detectors (Kamar products Inc., Zionsville, IN, USA), and electronic pressure sensors placed on the rump of cows such as HeatWatch II (CowChips, Manalapan, NJ, USA) (12). Devices to monitor secondary signs of oestrus, including pedometers and video recording with automated image analysis, have also been developed. Despite their efficiency and growing accuracy, performance has been hindered by parameter settings applied to these devices, such as threshold level, and outcomes have been affected by environmental conditions. High starting investment cost, estimated at €4,862 for the HeatWatch for a herd of 50 cows (which includes base station, software and monitors) is a further drawback for implementing these monitoring systems in dairy farms (13).

Biological measurements can provide an alternative strategy to mechanical and physical methods of oestrus detection, and efforts have been devoted to identifying suitable and easily accessible biomolecules in the cervico-vaginal mucus. These biomolecules can be monitored to provide an inexpensive means of rapidly and accurately assessing the onset of oestrus. Cervico-vaginal mucus physical characteristics, such as consistency and quantity, are altered in relation to the different stages of the oestrus cycle and in response to hormonal changes (14), with increased turbidity of the cervical mucus under the influence of progesterone as a recognised secondary sign of oestrus (15). These physical properties variation at oestrus may also be used as reference for oestrus detection (16). Cervico-vaginal mucus is a heterogeneous matrix produced by the secretory cells of the endocervix, and represents a very accessible source of discriminatory biomolecules for measurement (17). The mucus is primarily composed of water, ions, glycosaminoglycans, and glycoproteins which include mucins. Mucins are the major structural components of mucus and the majority of their mass is from *O*-linked oligosaccharides attached to the protein core which is responsible for the mucus' viscosity (18). Other glycoprotein components of mucus include immunoglobulin (Ig) Gs and lactoferrin (19), which all contribute to the overall mucus glycosylation profile. Glycosylation

of cervico-vaginal mucus is important because of its immunological function, modulation of the microbiota, and spermatozoa migration (20). The physical properties and composition of cervico-vaginal mucus is altered at oestrus in swine (21), ewes (22), and cows (23), and provides an environment suitable for reproduction (23). Abundances of proteins from the ovine cervico-vaginal tract vary at different time points of the oestrus cycle (24). As physical properties of mucus are highly dependent on glycosylation, it is likely that glycosylation is significantly altered during the oestrus cycle.

Detailed interrogation of mucins isolated from ovine and bovine reproductive tracts with carbohydrate-specific lectins previously indicated that cervico-vaginal glycosylation may be species- and breed-specific and may temporally vary with the oestrus cycle (25). However, isolation of mucins from mucus is a lengthy process, yielding a low amount of material and would be impractical for eventual field-based testing of individual animals. Thus, whole mucus provides a more convenient and economical source of material for analysis. Predictable, cyclical variation of the cervico-vaginal mucus glycosylation could offer temporally accurate biomarkers of the oestrus cycle associated with fertility using a conveniently sampled source to serve as an indicator for insemination and implantation with a higher likelihood of livestock conception.

Adaptation of whole mucus to the natural mucin microarray previously developed by our group facilitated a high throughput analysis of cervico-vaginal mucus glycosylation to identify possible trends in glycosylation associated with oestrus (25). The microarray platform offers multiplexed presentation of samples to facilitate rapid characterisation and comparison of carbohydrate structures presented in mucus. The objective of this proof of concept study was to investigate the possibility of using immobilised bovine cervico-vaginal mucus from a limited number of heifers, instead of purified mucins, to identify overall glycosylation changes over

parts of the oestrus cycle. Reliable oestrus biomarkers would facilitate more successful livestock fertilisation programmes.

2. Materials and Methods

2.1 Materials

Tetramethylrhodamine isothiocyanate (TRITC)-labeled lectins were purchased from EY Laboratories Inc. (San Mateo, CA, USA), the anti-MECA-79 (MECA79, rat IgM) antibody (200 µg/ml) was from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany) and the TRITC-labeled polyclonal goat anti-rat IgM secondary antibody (1 mg/mL) was from Bio-Rad (AbD Serotec, Oxford, UK). Nexterion® Slide H functionalised microarray slides were obtained from Schott AG (Jena, Germany). All other reagents were obtained from Sigma-Aldrich Co. (Dublin, Ireland), unless otherwise noted, and were of the highest grade available.

2.2 Bovine cervical mucus collection and processing

The oestrus cycle of three Limousin heifers were synchronised using a controlled internal drug release device (CIDR) (Eazi-Breed CIDR, Pfizer Animal health, Dublin, Ireland), with each CIDR impregnated with 1.38 g progesterone. Each animal had a CIDR inserted into the vagina and removed after 8 d. The day before CIDR removal, animals were treated with a prostaglandin analogue (Estrumate; Chanelle, Loughrea, Co. Galway, Ireland) to cause luteolysis. Cervico-vaginal mucus excretions were aspirated from live animals and sampling started 12 h post-CIDR removal. Mucus from the three animals was collected every 12 h over 4 d, and every 24 h for the following 5 d (Table 1). Animals were observed for the behavioural signs of heat (standing to be mounted by another animal) every 6 h from CIDR removal up to

82 h post-CIDR removal. All animals were expected to come into heat within 48-72 h post-CIDR removal. Time points presented were aligned to oestrus, which is used as the reference point. Sampling time points pre- and post- oestrus are labelled as O – hours and O + hours, respectively, with the number of hours reflecting hours between sampling (Table 1). Mucus was collected in 8 M guanidine hydrochloride (GuHCl), alkylated, and reduced as previously described (25), then lyophilised, and the weight of the final lyophilised powdered mucus recorded. All procedures were licensed by the Department of Health and Children, Ireland, in accordance with the Cruelty to Animals Act (Ireland 1897) and the European Community Directive 86/609/EC, and were sanctioned by the Animals Research Ethics Committee, University College Dublin, Ireland.

2.3 Construction of bovine cervico-vaginal mucus microarray

Lyophilised bovine cervico-vaginal mucus (Table 1) were diluted by weight in phosphate buffered saline, pH 7.4 (PBS) with appropriate concentration of Tween-20 (Supplementary Table 1) and printed onto Nexterion Slide H microarray slides using the piezoelectric non-contact dispensing sciFlEXARRAYER S3 (Scienion AG, Germany) equipped with a 90 µm uncoated glass nozzle at 62% humidity (± 2% tolerance) and constant temperature (18 °C) as previously described (25). Mucus samples were printed in replicates of six features, approximately 1 nL per feature (2 drops), with 8 replicate subarrays per microarray slide (Supplementary Figure 1). Microarray slides were incubated overnight at 18 °C in a high humidity chamber to complete conjugation. To deactivate any remaining functional groups on the microarray surface, microarrays were immersed in a solution of 100 mM ethanolamine and 50 mM sodium borate, pH 8.0, for 1 h at room temperature. Slides were washed three times in PBS supplemented with 0.05% Tween-20 (PBS-T) for 5 min each wash, then once in PBS.

Finally, the microarrays were centrifuged dry (475 x *g*) and stored at 4 °C with desiccant. Microarrays were used within 4 months of construction.

2.4 Glycoprofiling of bovine cervico-vaginal mucus

Incubations were carried out essentially as previously described (25). In brief, a panel of TRITC-labelled lectins (Table 2) were diluted in Tris-buffered saline (TBS; 20 mM Tris-HCl, 100 mM NaCl, 1 mM $CaCl_2$, $MgCl_2$, pH 7.2) with 0.05% Tween-20 (TBS-T). Seventy µl of each lectin dilution was applied to a corresponding well of an eight-well gasket slide, enclosed in an incubation cassette system (Agilent Technologies, Cork, Ireland) and incubated for 1 h at 23 °C with gentle inversion (4 rpm). Slides were then washed twice in TBS-T and once in TBS prior to centrifuging dry for 5 min at 475 x *g*. Slides were scanned immediately in an Agilent G2505 microarray scanner (Agilent Technologies, Cork, Ireland) (532 nm laser, 90% PMT, 5 µm resolution) and images were saved as tagged image (.tif) files. Lectin concentrations were initially titrated (5 to 20 µg/mL) and the concentration giving the best signal-to-background ratio for each was selected (Table 2).

For inhibition experiments, each TRITC-labelled lectin was diluted to the same concentration in respective 100 mM solution of inhibitory carbohydrate (Table 2) in TBS-T and incubated in parallel on different subarrays on the same slide as the uninhibited sample. Resulting binding intensity was compared to the binding intensity resulting from incubation with no inhibitor (Supplementary Table 2). A lectin was considered to bind to a mucus sample if the relative fluorescence was greater than 1,000 relative fluorescence units (RFU) (i.e. five times background (26) and/or binding was inhibitable by ≥40% with corresponding inhibitory carbohydrate.

Similarly, MECA79 antibody was incubated at a 1 in 100 dilution in TBS-T on the mucin microarray slide for 1 h, washed and dried as above, and then immediately incubated with TRITC-labelled polyclonal goat anti-rat IgM (1 in 500 dilution in TBS-T). Microarray slides were washed and dried as above and scanned immediately. Control consisted of incubating with PBS instead of the primary antibody and no fluorescence was noted for secondary antibody alone (not shown).

2.5 Data extraction and analysis

Fluorescence intensity values were extracted from microarray images using GenePix Pro v.6.1.0.4 and a proprietary *.gal file using adaptive diameter (70-130 %) circular alignment based on 230 μm features and exported as text to Excel (version 2010, Microsoft) for data analysis. Median feature intensities with local background subtracted (F532median-B532) were used for each feature intensity value. The median of six replicate features per subarray was handled as a single data point for graphical and statistical analysis and considered as one experiment. Data were normalised to the mean total intensity per subarray for experimental (technical) replicates (n=3, or n=2 in specified cases). Unsupervised hierarchical clustering of lectin and antibody binding intensity data was performed with Hierarchical Clustering Explorer v3.0 (http://www.cs.umd.edu/hcil/hce/hce3.html). The mean intensity data for each mucus sample was rescaled within 0 to 65,000 RFU and was clustered with the following parameters: no pre-filtering, complete linkage, and Euclidean distance. The significance of inhibition data was evaluated using a standard Student's t test (paired, two-tailed).

3. Results and discussion

3.1 Cervico-vaginal mucus collection and optimised mucus microarray printing

The CIDR was inserted and removed at the same time for the 3 animals and sampling started 12 h following CIDR removal. Samples were taken every 12 h for 4 d following removal and every 24 h thereafter for another 5 d (Table 1) for all animals. Oestrus was detected by observation 36 h following CIDR removal for cows 685 and 673, close to the typical 48-72 h post-CIDR removal, and 84 h post-CIDR removal for cow 664, which is later than normal.

Lyophilised mucus was resuspended in PBS and directly printed onto amine-reactive, *N*-hydroxylsuccinimide (NHS) ester-functionalised hydrogel Nexterion Slide H surfaces at physiological pH 7.4. This method was successfully used previously for purified mucin conjugation (25) and also enables 3D presentation of the immobilised biological components, thus maintaining their biologically-relevant presentation. Similar to previous reports requiring individual optimisation of printing conditions for samples of different viscosities (25, 27), both mucus (0.25 to 1 mg/ml, by weight) and detergent concentration (0.025 to 0.05% Tween 20) in the print buffer were individually optimised for printing each cervico-vaginal mucus sample (Supplementary Table 1). The resulting printed features as visualised by images of the lectin incubated microarrays were assessed and final mucus print concentration and detergent content was determined based on feature formation on the microarray (sample was printed or not, and coverage of the printed feature) and extractable feature quality (Supplementary Figure 1). Throughout the study, lectin binding on the O + 36 h mucus sample for cow 664 was consistently low, suggesting a missed print. However, this sample was plotted in the presented graphs to facilitate comparisons between the three animals (Figure 1).

3.2 Mucus glycosylation profiles

Glycosylation changes over part of the oestrus cycle were investigated by profiling printed mucus on the microarray with nine fluorescently labelled lectins covering a broad range of carbohydrate motifs (Table 2) and an antibody against the 6-sulfo-sialyl Lewis x (6-sulfo-

SLex) structure, MECA79 (Figure 1, Supplementary Figures 2-5). The lectin binding profile was unique for each animal (Figure 1, Supplementary Figures 2-5). To confirm carbohydrate-mediated binding of the lectins the labelled lectins were co-incubated with their haptenic sugars to assess binding inhibition (Table 2 and Supplementary Table 2) (28). The data for LTA was subsequently disregarded as this lectin did not qualify as carbohydrate-mediated binding to the mucus samples, with LTA binding intensities below 1,000 RFU and no inhibition with fucose (Fuc) (Supplementary Table 2). To directly compare glycosylation changes around oestrus between animals, time points were aligned at oestrus detected (O detected, Table 1) and changes between 24 h pre oestrus (O – 24) observed and 36 h post oestrus observed (O + 36) will be discussed.

Changes in lectin and antibody binding intensities from 24 h before detected oestrus until 36 h post-oestrus indicated changes in the cervico-vaginal mucus glycosylation in each animal during the oestrus cycle (Figure 1, Supplementary Figure 3). Cows 673 and 685 displayed the greatest similarities in overall binding pattern, with higher lectin binding intensities before oestrus, whereas lectin binding increased for cow 664 after oestrus (Figure 1, Supplementary Figure 3). It is important to point out that all mucus samples were not printed at the same concentration, although all print concentrations ranged between 0.25 to 1 mg/mL. However the changes in lectin binding intensity did not correlate with an increase in mucus printing concentration but did vary over time. Variations in sialylation (indicated by MAA and SNA-I binding), fucosylation (UEA-I and AAA), sialyl T-antigen (MAA and AIA with low PNA binding), high mannose *N*-linked structures (Con A) and terminal *N*-acetylgalactosamine (GalNAc) residues (WFA) and sulfated structures (WFA and MECA79 antibody binding) were observed across time points. PNA, AAA and MECA79 antibody showed very low binding intensities (<1,000 RFU) and the least binding variation over the time points (Figure 1). MECA79 antibody binding depends upon the presence of carbohydrate and sulfated epitopes

and it recognises sulfated carbohydrate epitopes, including 6-sulfo-*N*-acetylglucosamine (GlcNAc-6-SO_4), part of 6-sulfo-SLex, and 6-sulfolactose (29) (Table 2), which are all ligands of L-selectin. These ligands play an important part in fertility and embryo implantation in humans (30), although this may be more relevant for the uterine and fallopian epithelial cells rather than mucus (30-32). Low binding of MECA79 to bovine cervical mucin was shown in a previous study (25). The low intensity and subtle variation in MECA79 binding across time may indicate that the motif is expressed at a low level in bovine cervico-vaginal mucus (i.e. on mucins and/or other glycoproteins) when fertilisation has not occurred.

Alterations to AAA and UEA-I binding over the time course of mucus sampling were noted for all three cows (Figure 1) and indicated the presence of α-(1→2)-linked fucosylation (UEA-I, Table 2) and possibly additionally α-(1→3)-linked fucosylation or the Lewis a structure (33) (AAA, Table 2). This α-(1→2)-linked Fuc structure (H antigen) within reproductive mucus was previously demonstrated as the most abundant Fuc linkage in mice by LC-MS analysis (34), and the overall quantity of Fuc is lower at oestrus compared to the rest of the reproductive cycle (23). Fuc residues have been shown to mediate the binding of bull sperm onto oviductal epithelium for reservoir formation following insemination, especially in α-(1→4) linkage to GlcNAc, such as in the Lewis a trisaccharide (35). Mass spectrometry (MS) analysis of carbohydrates released from humans cervical mucus at ovulation showed an increase in fucosylation and sialylation before and after ovulation (36).

The binding of PNA, specific for the T antigen in the absence of sialylation (Table 2), was low for all animal mucus samples at every time point and no significant variation was observed over the sampling period. The presence of T antigen has been shown on cervical epithelial cells by PNA staining in a previous study (37), and variation in T antigen expression coinciding with an increase in MUC5B secretion in cervico-vaginal mucus was also shown in humans (38). Binding of AIA, also specific for the T antigen but capable of binding to T antigen even if the

disaccharide is sialylated (Table 2), was also noted. SNA-I and MAA binding, which indicated the presence of terminal α-(2→6)- and α-(2→3)-linked sialic acid, respectively (Table 2), along with AIA binding suggested that the accessible carbohydrate motifs in bovine cervico-vaginal mucus were sialyl T antigens. This correlates with previous findings from Kilcoyne, *et al.* (25).

Con A binding indicated the presence of mannose (Man) residues on N-linked oligosaccharides such as high mannose structures and terminal Man and Man core structures in *N*-linked oligosaccharides (Table 2), which are present on mucins in low abundance compared to O-linked oligosaccharides and on other glycoproteins in cervico-vaginal mucus such as IgGs and lactoferrin. High binding intensity to mucus samples was observed with WFA and MAA throughout the sampling period. Binding of WFA suggested the presence of GalNAc residues (which may be sulfated) (Table 2) in cervico-vaginal mucus.

The above observations were consistent between the 3 animals, indicating alterations in GalNAc, sialylated and possibly sulfated motifs over oestrus and/or differences in their presentation and accessibility. An increase in sulfated cervical mucins at oestrus has been demonstrated in previous studies (25, 37) as well as an increase in sialylation at oestrus (37, 39). This may be due to an increase in sulfated glycosaminoglycans on proteoglycans at oestrus (40), such as chondroitin sulfate, known to promote sperm capacitation in the female reproductive tract (41) or sulfated carbohydrate epitopes on glycoproteins. An increase in SNA-I binding prior to oestrus, suggesting an increase in expression of terminal α-(2→6)-linked sialic acid, is in agreement with Hashiba, *et al.* (42), who showed that the increase in α-(2→6)-linked sialylation played an important role in luteolysis. The increase in terminal α-(2→6)-linked sialylation contribution to luteolysis in cows is thought to correlate with a decrease in galectin-1 binding, which was shown to have a protective effect on luteal cell viability (43). Terminal sialic acid is also thought to contribute to lower sperm transport through the cervix where a higher concentration was found in ewe breed with lower pregnancy rate (44).

3.3 Oestrus-associated mucus glycosylation

Glycosylation trends at oestrus using 6 time points around oestrus (including oestrus) were examined by hierarchical clustering to identify trends in glycosylation pattern between the 3 animals (Figure 1 and 2(A)). Binding of all lectins to mucus collected from cow 664 was generally less intense than with mucus from the other two (Supplementary Figure 5), therefore data for each mucus sample were scale-normalised to a 65,500 RFU maximum to remove any potential artefacts generated from intensity differences rather than genuine pattern differences (Figure 2(A)). For each animal, reproductive mucus had a glycosylation profile unique to oestrus and also the animal. Lectins also clustered according to their individual relative binding intensity patterns across all mucin samples. SNA-I, WFA, ConA and MAA demonstrated relatively higher binding affinity across the sample set than MECA79, AAA, UEA-I, PNA and AIA resulting in each of these sets of lectins being clustered together. SNA-I and WFA clustered together suggesting a similar binding pattern around oestrus. High lectin binding at oestrus might be related to increased expression of the relevant carbohydrate structures at oestrus but may also be due to higher mucin production overall from higher mucin gene transcription as previously described in ewes (24).

Glycosylation changes in mucus secretions around oestrus highlighted a lectin binding pattern specific to oestrus for each animal (Figure 2(A) and (B)) which could be exploited to pinpoint each animal's highest fertility days. Monitoring of the physical properties of cervical mucus is of value to potentially pinpoint highest fertility days in humans (45), buffalos (15) and cows (19), however this could be narrowed down to hours using by assessing the glycosylation changes in cervico-vaginal mucus. Matrix plot analysis was carried out with lectins and MECA79 antibody to examine the grouping of time points according to their lectin binding and find a recognition molecule pair that could most clearly discriminate time points around oestrus and therefore be used as potential trends. Thirty-six pairs were analysed in total and the

majority of pairings followed a similar pattern with grouping at oestrus, especially for cows 673 and 685, confirming the outcome for HCE analysis (Figure 2, Supplementary Figures 6-14). GalNAc with possible sulfation and increased sialylation as detected by WFA and SNA-I binding, respectively, confirmed the importance of these structures at oestrus making these recognition molecules potentially effective for detection of oestrus (Figure 2(B)) when used together. However, these observations were not limited to SNA-I and WFA but to most pairs that were investigated (Supplementary Figure 6-14).

These observations did not apply to cow 664, suggesting that oestrus probably occurred at an earlier time point than true onset. The glycosylation pattern for cow 664 followed a different trend when compared to the other two animals for most lectins (Figure 2(B)). Oestrus cycling in the 3 animals was synchronised with the insertion of CIDR and oestrus was detected by observation between 24 h and 48 h following CIDR removal for cows 685 and 673, and over 80 h following removal for cow 664 (Table 1), which may explain the discrepancy between glycosylation patterns between cows 664 and the other two cows. It is possible that earlier visual cues for heat detection in animal 664 were missed or cow 664 may not have exhibited signs of oestrus, also called silent heat, which has been increasing in dairy farming over recent years (46). Similarities in glycosylation profile at oestrus detected (shown in Figure 2(B), Supplementary Figures 6-14 left column) were therefore examined to test the latter hypothesis. Matrix plot analysis was carried for WFA and SNA-I binding and oestrus time points for cows 673 and 685 and all sampling time points until oestrus detected for cow 664 (Figure 3). O-60 (oestrus minus 60 h) grouped with the oestrus mucus samples for animals 673 and 685. The same analysis was carried out with all lectin pair combinations and in all cases oestrus 673 and 685 grouped with O-60 (Supplementary Figures 6-14, right column), suggesting that oestrus probably occurred for cow 664 at O-60. Taken together, these data suggests that visual signs

of oestrus for cow 664 may have been missed, confirming the need for more accurate oestrus detection than visual signs.

Taken together, these data suggest that cervico-vaginal mucus glycosylation changes during oestrus and that a unique mucus glycosylation pattern could be associated with oestrus. This unique glycosylation was identified by a combination of relative binding of SNA-I and WFA and could be exploited to more accurately identify fertilisation times compared to visual signs.

4. Conclusions

Cervico-vaginal secretions represent a readily accessible sample matrix for diagnostic testing using simple swabbing and therefore identification of changes over the oestrus cycle is of potential utility as a convenient and precise biomarker for oestrus. The temporal alteration of whole cervico-vaginal mucus glycosylation from three cows was monitored using fluorescently labelled lectins and an antibody in a microarray format. A difference in mucus glycosylation at oestrus compared to the rest of the cycle was identified and could be discriminated using a combination of the lectins SNA-I and WFA. The observed trends could serve as a starting point for a validation study involving a larger number of animals and sampling points over the whole oestrus cycle to potentially implement glycosylation-based biomarkers as indicators of oestrus. In turn, such lectins or specific recognition molecules could be incorporated into rapid, field-deployable tests for oestrous detection in individual cows facilitating more successful reproduction management programmes.

Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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Table 1. Time points for collection of cervico-vaginal mucus secretions from three heifers (685, 673 and 664) and events in time (h) related to oestrus (O = oestrus, detected by observation of mounting behaviour). N.s., no sample collected.

Sampling day	**Sampling number**	**Sampling time**	**Event 685**	**Event 673**	**Event 664**
0	n.s.	0800	CIDR out	CIDR out	CIDR out
0	1	2000	O - 24	O - 24	O - 72
1	2	0800	O - 12	O - 12	O - 60
1	3	2000	O detected	O detected	O - 48
2	4	0800	O + 12	O + 12	O - 36
2	5	2000	O + 24	O + 24	O - 24
3	6	0800	O + 36	O + 36	O - 12
3	7	2000	O + 48	O + 48	O detected
4	8	0800	O + 56	O +56	O + 12
4	9	0800	O + 72	O + 72	O + 24
5	10	0800	O + 84	O + 84	O + 36
6	11	0800	O + 108	O + 108	O + 60
7	12	0800	O + 132	O + 132	O + 84
8	13	0800	O + 156	O + 156	O + 108
9	14	0800	O + 180	O + 180	O + 132

Table 2. Antibody and lectins used for mucus glycoprofiling, their binding specificities, concentrations used, and inhibitory carbohydrate (100 mM).

Carbohydrate recognition molecule	Abbreviation	Major binding specificity	Conc. µg/mL	Inhibitory carb.
Anguilla anguilla lectin	AAA	α-(1→3)- and α-(1→2)-linked Fuc, Lewis a	10	Fuc
Artocarpus integrifolia agglutinin	AIA	Gal, Gal-α-(1→3)-GalNAc (T-antigen), α-(1→6)-linked Gal, sialylation independent	15	Gal
Canavalia ensiformis (jack bean lectin)	Con A	α-linked Man>Glc>GlcNAc	10	Man
Lotus tetragonolobus lotus lectin	LTA	α-(1→3)-linked Fuc	10	Fuc
Maackia amurensis agglutinin	MAA	Neu-α-(2→3)-Gal = Gal-3-SO_4 >Lac	15	Lac
MECA79 antibody	MECA79	GlcNAc-6-SO_4 as part of the 6-sulfo-sialyl Lewis x (6-sulfo-SLex), 6-sulfolactose	10	n.a.
Arachis hypogaea peanut agglutinin	PNA	Gal (Gal-ß-(1→3)-GalNAc (T-antigen) >GalNAc>Lac>Gal, terminal ß-Gal)	10	Gal
Sambucus nigra agglutinin I	SNA-I (SNA)	Neu-α-(2→6)-Gal(NAc) >Lac, GalNAc >Gal	10	Lac
Ulex europaeus agglutinin I	UEA-I	α-(1→2)-linked Fuc, H type 2 antigen	10	Fuc
Wisteria floribunda agglutinin	WFA	GalNAc (GalNAc-α-(1→6)-Gal>GalNAc-α-(1→3)-R GalNAc (Forsmann antigen) >GalNAc>>Lac>Gal, chondroitin sulfate	10	Gal

Figure 1. Glycosylation profiles of bovine cervico-vaginal mucus at different time points around oestrus detected following oestrus induction in cows (A) 664, (B) 673 and (C) 685. Bars represents the average binding intensity of fluorescently labelled lectins and MECA-79 antibody to printed mucus from three replicates experiments (in duplicate for MECA79, SNA-I, Con A and MAA) and error bars represent +/- 1 standard deviation.

Figure 2. (A) Heat map and dendrograms for bovine cervico-vaginal mucus glycosylation profiles generated using labelled lectins and MECA79 antibody. Mean intensity data for each mucus sample was scaled 0-65,500 RFU and subjected to unsupervised clustering with Euclidean distance and complete linkage. Six time points around oestrus characterized by relatively high intensity binding with the majority of lectins were grouped together. (B) Matrix plot analysis of WFA and SNA binding behavior with bovine cervico-vaginal mucus showing grouped time points at oestrus (blue oval).

Figure 3. (A) Matrix plot analysis of WFA and SNA-I binding to bovine cervico-vaginal mucus. Sampling point up to observed oestrus were selected for cow 664 and oestrus binding data selected for cows 673 and 685. Time points with similar lectin binding pattern were grouped (blue oval).

Figure 1 (in colour)

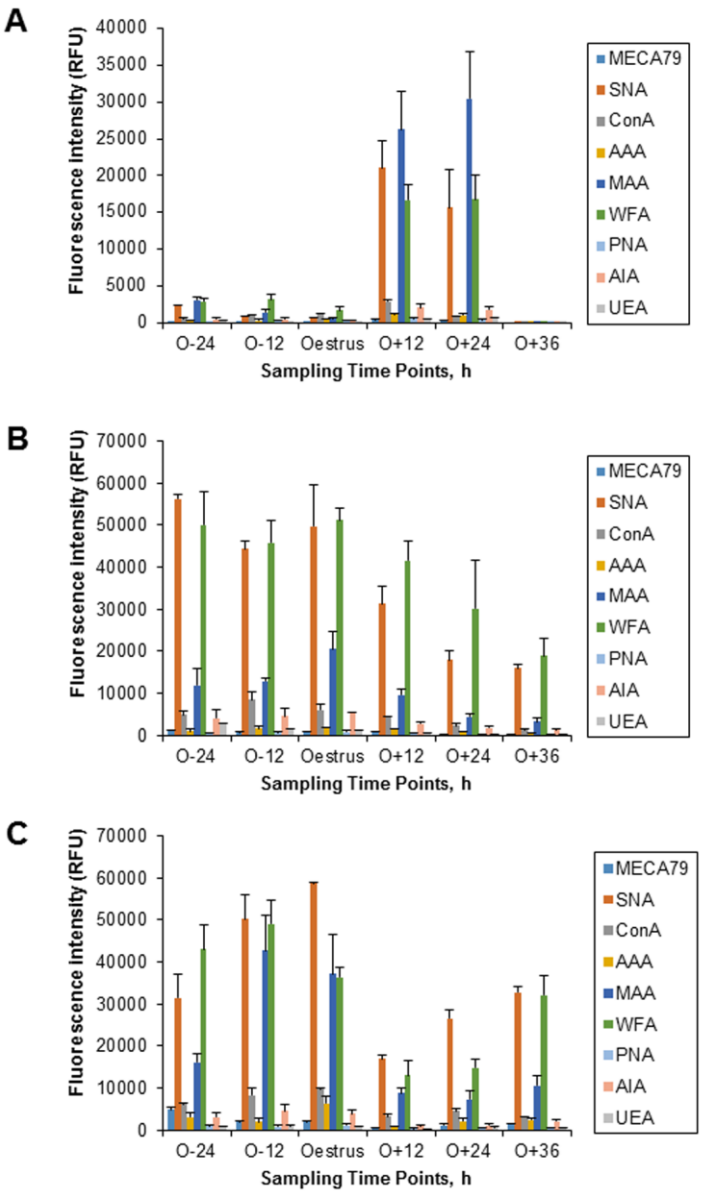


Figure 2 (in colour)

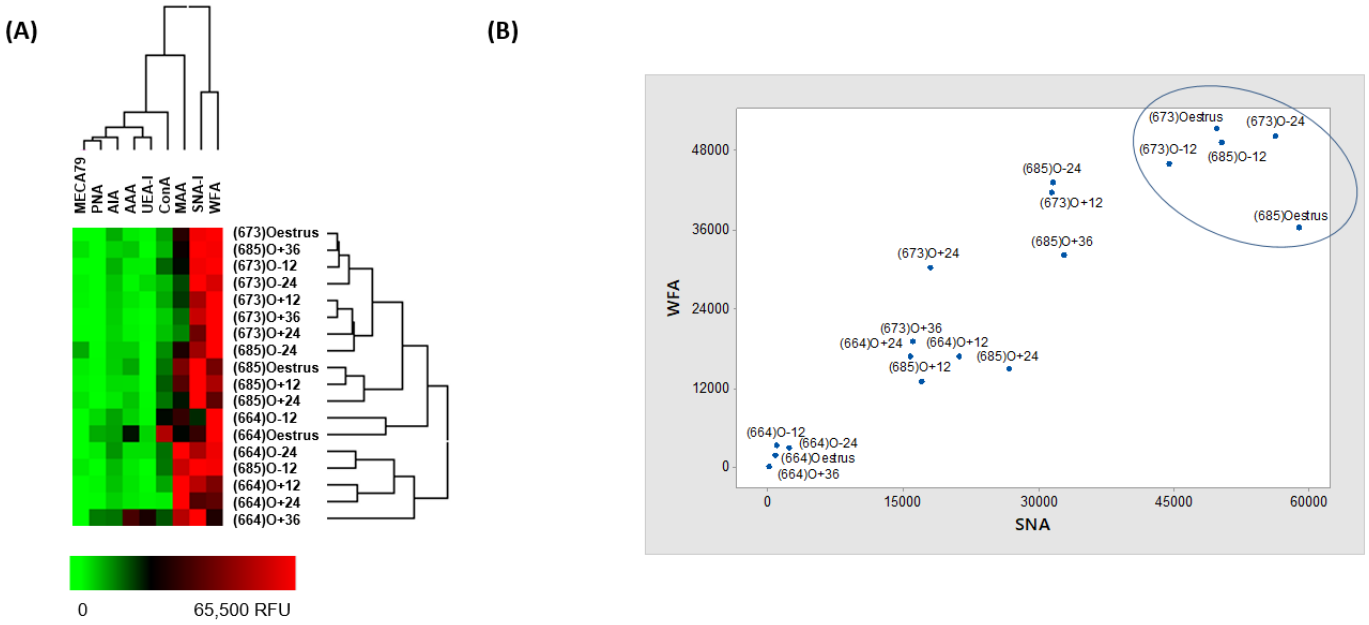
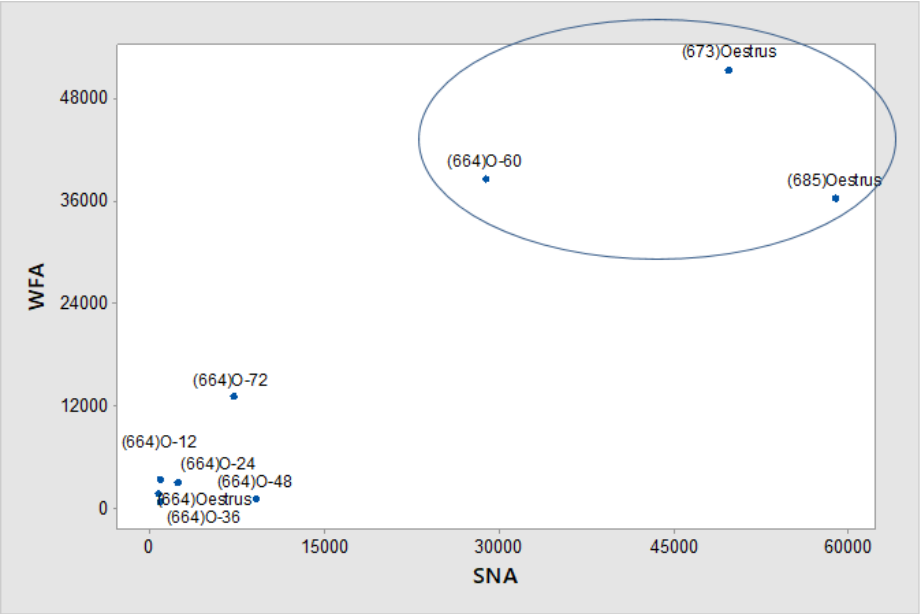


Figure 3


(673)Oestrus
48000
(664)O-60
(685)Oestrus
36000
WFA
24000
(664)O-72
12000
(664)O-12
(664)O-24
(664)O-48
(664)Oestrus
0
(664)O-36
0
15000
30000
45000
60000
SNA

Supplementary information

Examination of the oestrus-dependent alterations of bovine cervico-vaginal mucus glycosylation potential for determining optimum timing of fertilisation

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* Joint corresponding authors

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(C) 685, zoom-in on lectin binding intensity below 8,000 RFU. Bars represents the average binding intensity of fluorescently labelled lectins and MECA-79 antibody to printed mucus from three replicates experiments (in duplicate for MECA79, SNA-I, Con A and MAA) and error bars represent +/- 1 standard deviation.

Supplementary Figure 3. Glycosylation profiles of bovine cervico-vaginal mucus at different time points following oestrus induction in cow 685 and relationship to sampling time points. (A) Bars represents the average binding intensity of fluorescently labelled lectins and MECA-79 antibody to printed mucus from three replicates experiments (in duplicate for MECA79, SNA, Con A and MAA) and error bars represent +/- 1 standard deviation. (B) Zoom-in on lectin binding intensity below 8,000 RFU (C) Sampling time line describing relationship between sampling number, sampling day and oestrus observed.

Supplementary Figure 4. Glycosylation profiles of bovine cervico-vaginal mucus at different time points following oestrus induction in cow 673 and relationship to sampling time points. (A) Bars represents the average binding intensity of fluorescently labelled lectins and MECA-79 antibody to printed mucus from three replicates experiments (in duplicate for MECA79, SNA, Con A and MAA) and error bars represent +/- 1 standard deviation. (B) Zoom-in on lectin binding intensity below 8,000 RFU (C) Sampling time line describing relationship between sampling number, sampling day and oestrus observed.

Supplementary Figure 5. Glycosylation profiles of bovine cervico-vaginal mucus at different time points following oestrus induction in cow 664 and relationship to sampling time points. (A) Bars represents the average binding intensity of fluorescently labelled lectins and MECA-79 antibody to printed mucus from three replicates experiments (in duplicate for MECA79, SNA, Con A and MAA) and error bars represent +/- 1 standard deviation. (B) Zoom-in on lectin binding intensity below 8,000 RFU (C) Sampling time line describing relationship between sampling number, sampling day and oestrus observed.

Supplementary Figure 6. Matrix plot analysis of lectins binding onto mucus microarray showing grouped time points at oestrus observed (left column) and potentially oestrus detected (right column).

Supplementary Figure 7. Matrix plot analysis of lectins binding onto mucus microarray showing grouped time points at oestrus observed (left column) and potentially oestrus detected (right column).

Supplementary Figure 8. Matrix plot analysis of lectins binding onto mucus microarray showing grouped time points at oestrus observed (left column) and potentially oestrus detected (right column).

Supplementary Figure 9. Matrix plot analysis of lectins binding onto mucus microarray showing grouped time points at oestrus observed (left column) and potentially oestrus detected (right column).

Supplementary Figure 10. Matrix plot analysis of lectins binding onto mucus microarray showing grouped time points at oestrus observed (left column) and potentially oestrus detected (right column).

Supplementary Figure 11. Matrix plot analysis of lectins binding onto mucus microarray showing grouped time points at oestrus observed (left column) and potentially oestrus detected (right column).

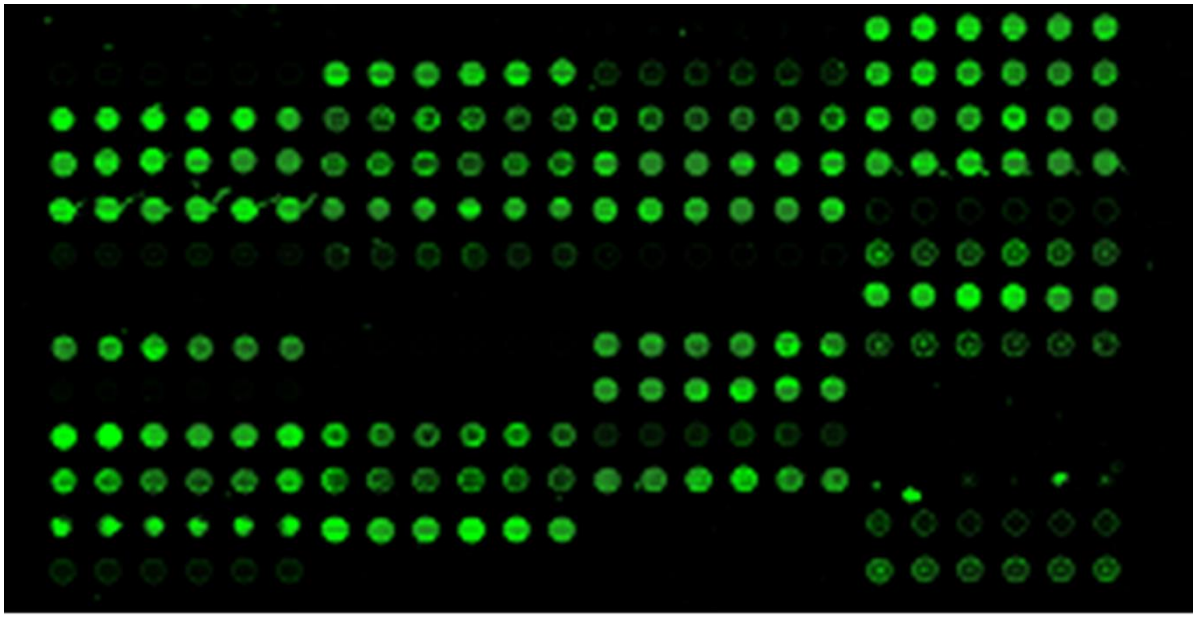
Supplementary Figure 12. Matrix plot analysis of lectins binding onto mucus microarray showing grouped time points at oestrus observed (left column) and potentially oestrus detected (right column).

Supplementary Figure 13. Matrix plot analysis of lectins binding onto mucus microarray showing grouped time points at oestrus observed (left column) and potentially oestrus detected (right column).

Supplementary Figure 14. Matrix plot analysis of lectins binding onto mucus microarray showing grouped time points at oestrus observed (left column) and potentially oestrus detected (right column).

Supplementary Table 1. Bovine cervical mucus, print information and concentrations used. Print buffer was PBS, pH 7.4, supplemented with varying percentages of Tween-20 indicated by % T.

Cow	Sampling number	Print buffer % T	Mucus conc (mg/mL)
664	1	PBS 0.025% T	0.9
664	2	PBS 0.025% T	0.5
664	3	PBS 0.025% T	0.9
664	4	PBS 0.025% T	0.9
664	5	PBS 0.025% T	0.9
664	6	PBS 0.025% T	0.9
664	7	PBS 0.025% T	0.9
664	8	PBS 0.025% T	0.9
664	9	PBS 0.05% T	0.5
664	10	PBS 0.05% T	0.9
664	11	PBS 0.025% T	0.9
664	12	PBS 0.025% T	0.9
664	13	PBS 0.025% T	0.5
664	14	PBS 0.025% T	0.25
685	1	PBS 0.025% T	0.25
685	2	PBS 0.025% T	0.75
685	3	PBS 0.025% T	0.5
685	4	PBS 0.05% T	1
685	5	PBS 0.05% T	0.25
685	6	PBS 0.05% T	0.5
685	7	PBS 0.025% T	1
685	8	PBS 0.05% T	0.5
685	9	PBS 0.025% T	1
685	10	PBS 0.025% T	0.5
685	11	PBS 0.025% T	1
685	12	PBS 0.025% T	0.5
685	13	PBS 0.025% T	0.5
685	14	PBS 0.05% T	1
673	1	PBS 0.025% T	0.5
673	2	PBS 0.05% T	0.75
673	3	PBS 0.05% T	0.75
673	4	PBS 0.05% T	0.75
673	5	PBS 0.025% T	0.9
673	6	PBS 0.05% T	0.9
673	7	PBS 0.025% T	0.9
673	8	PBS 0.025% T	0.9
673	9	PBS 0.025% T	0.5
673	10	PBS 0.05% T	0.9
673	11	PBS 0.05% T	0.9
673	12	PBS 0.025% T	0.25
673	13	PBS 0.05% T	0.9
673	14	PBS 0.05% T	0.9



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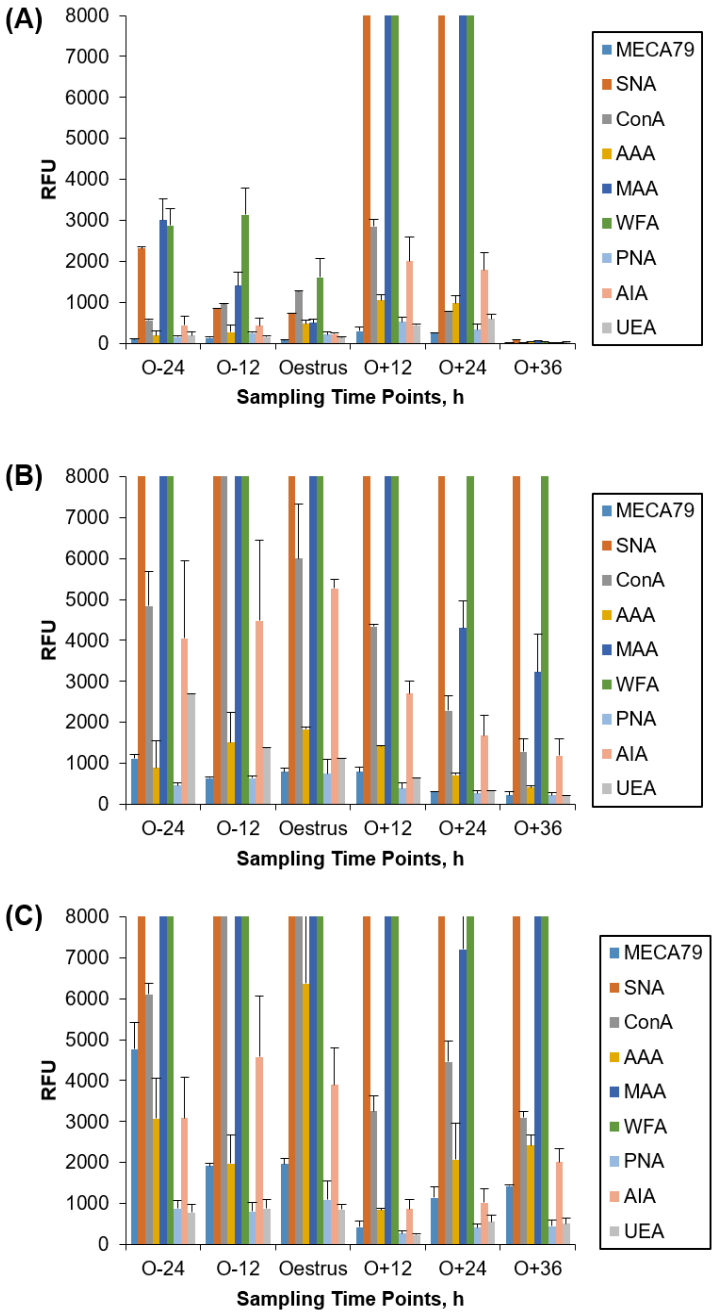

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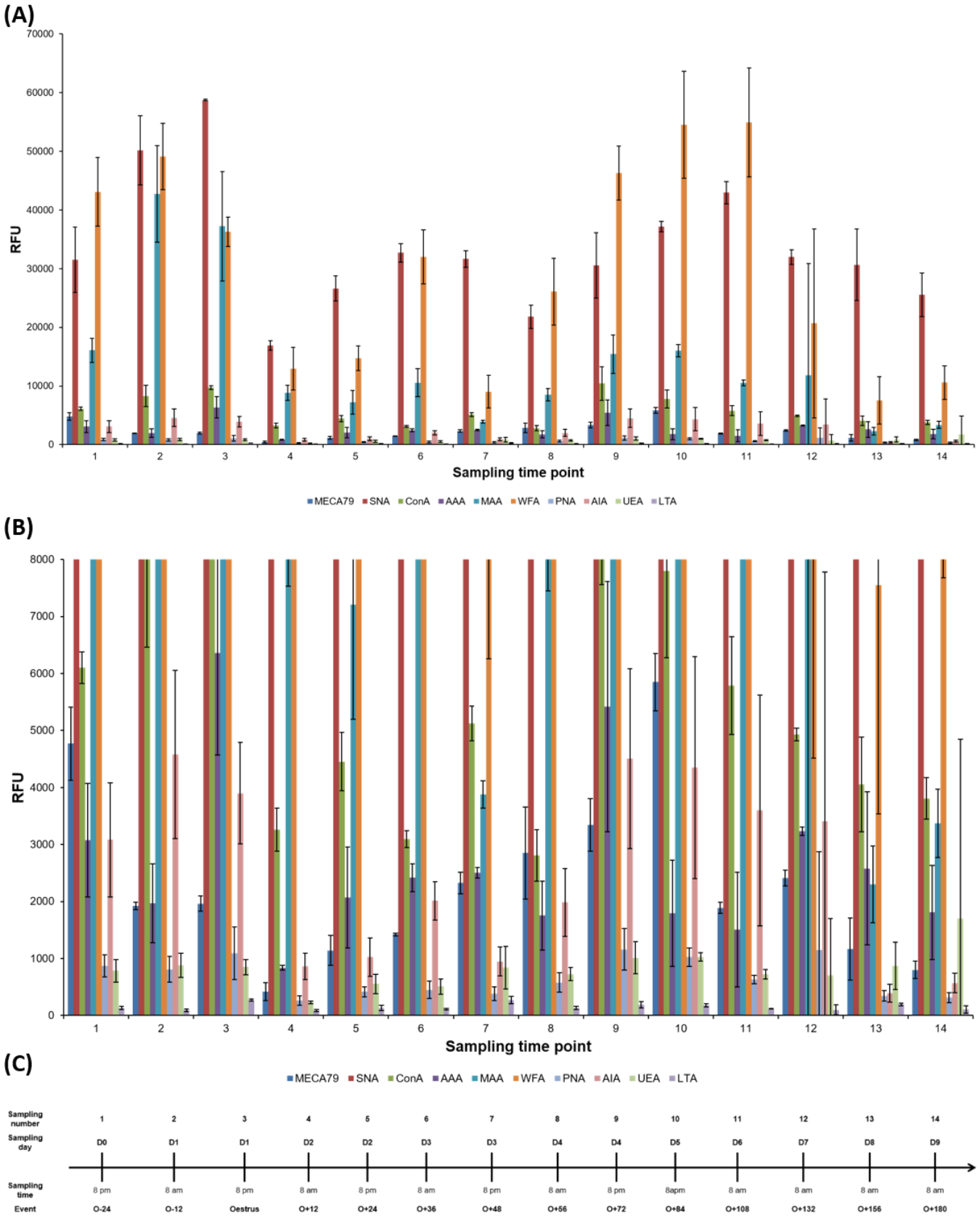

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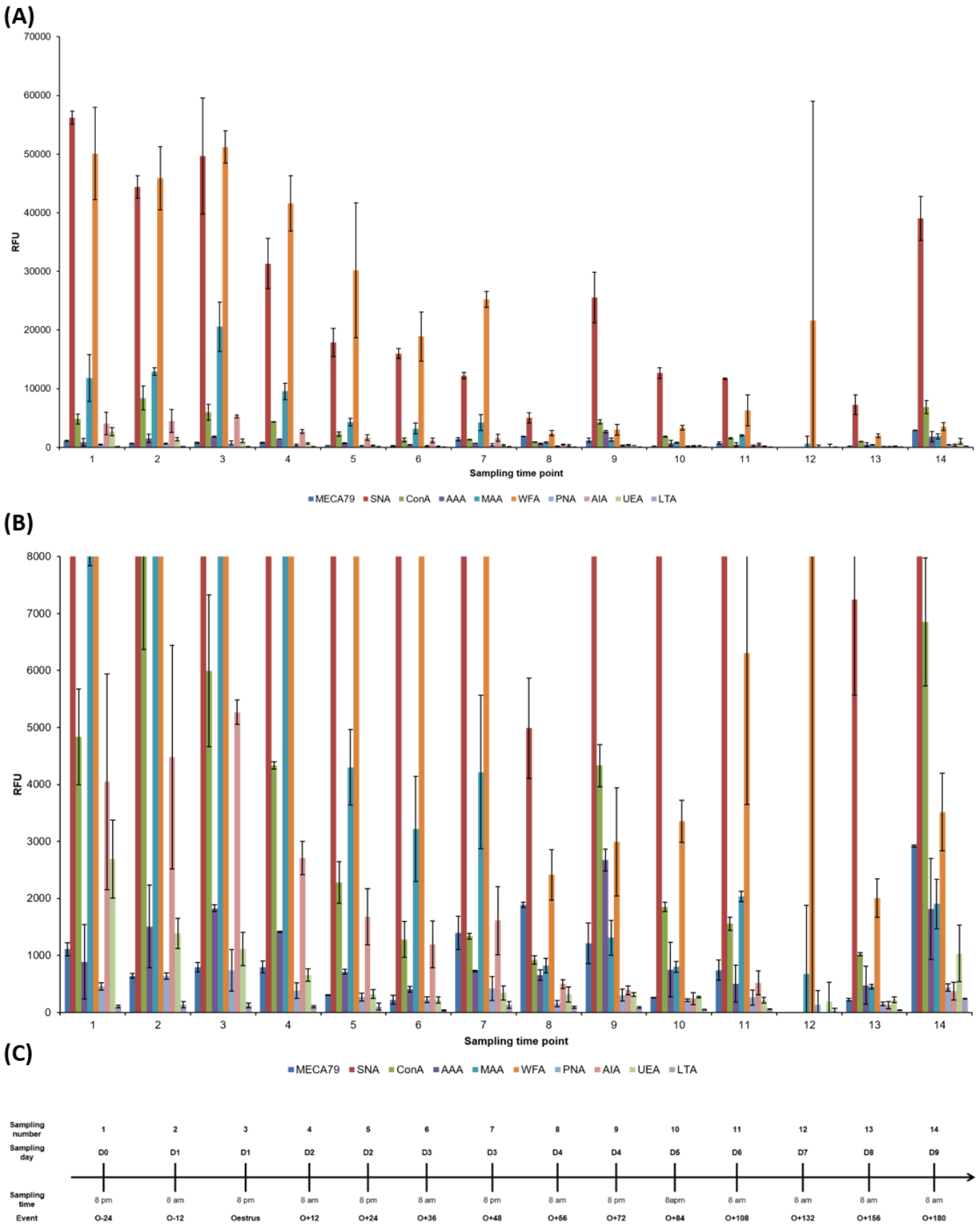

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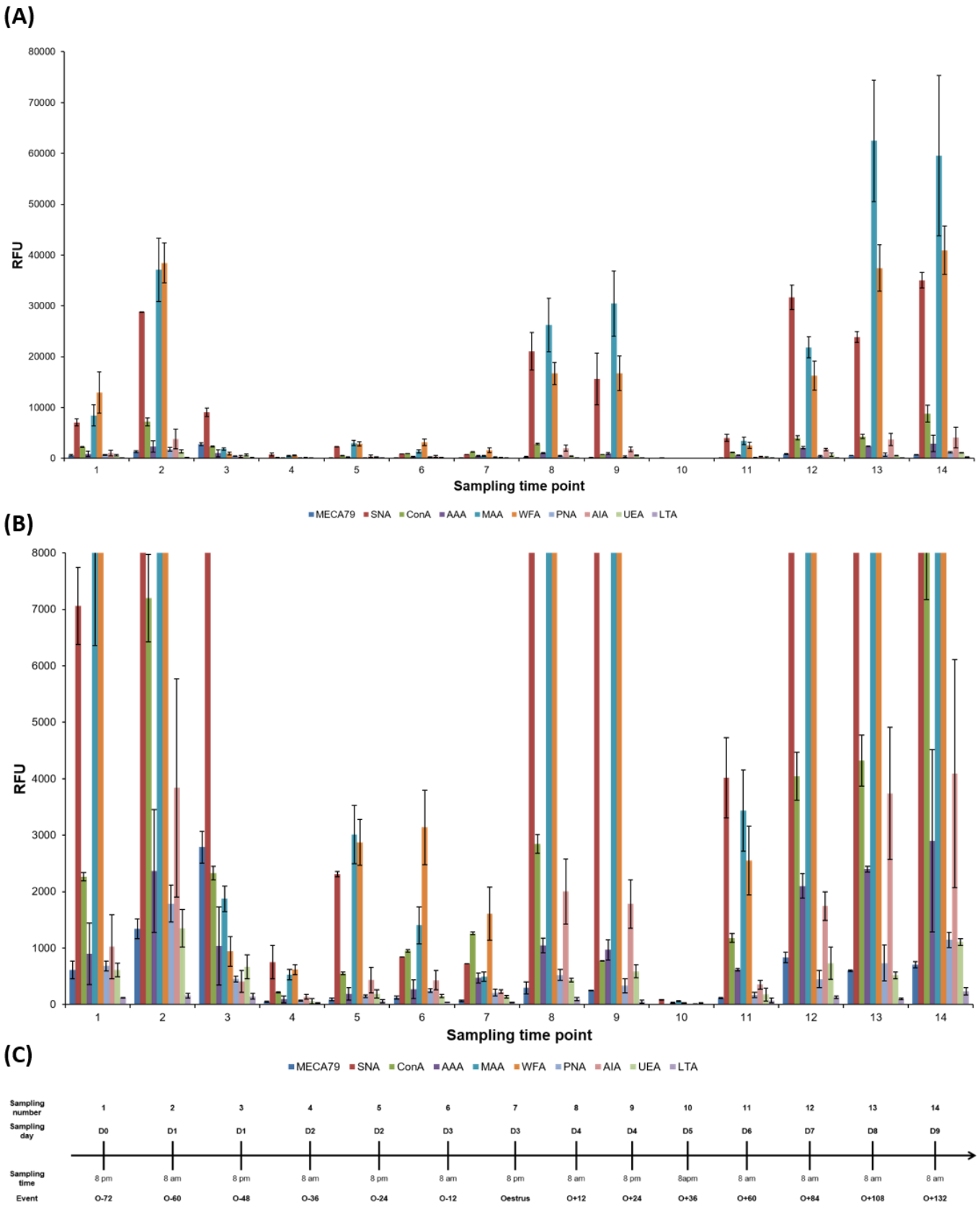

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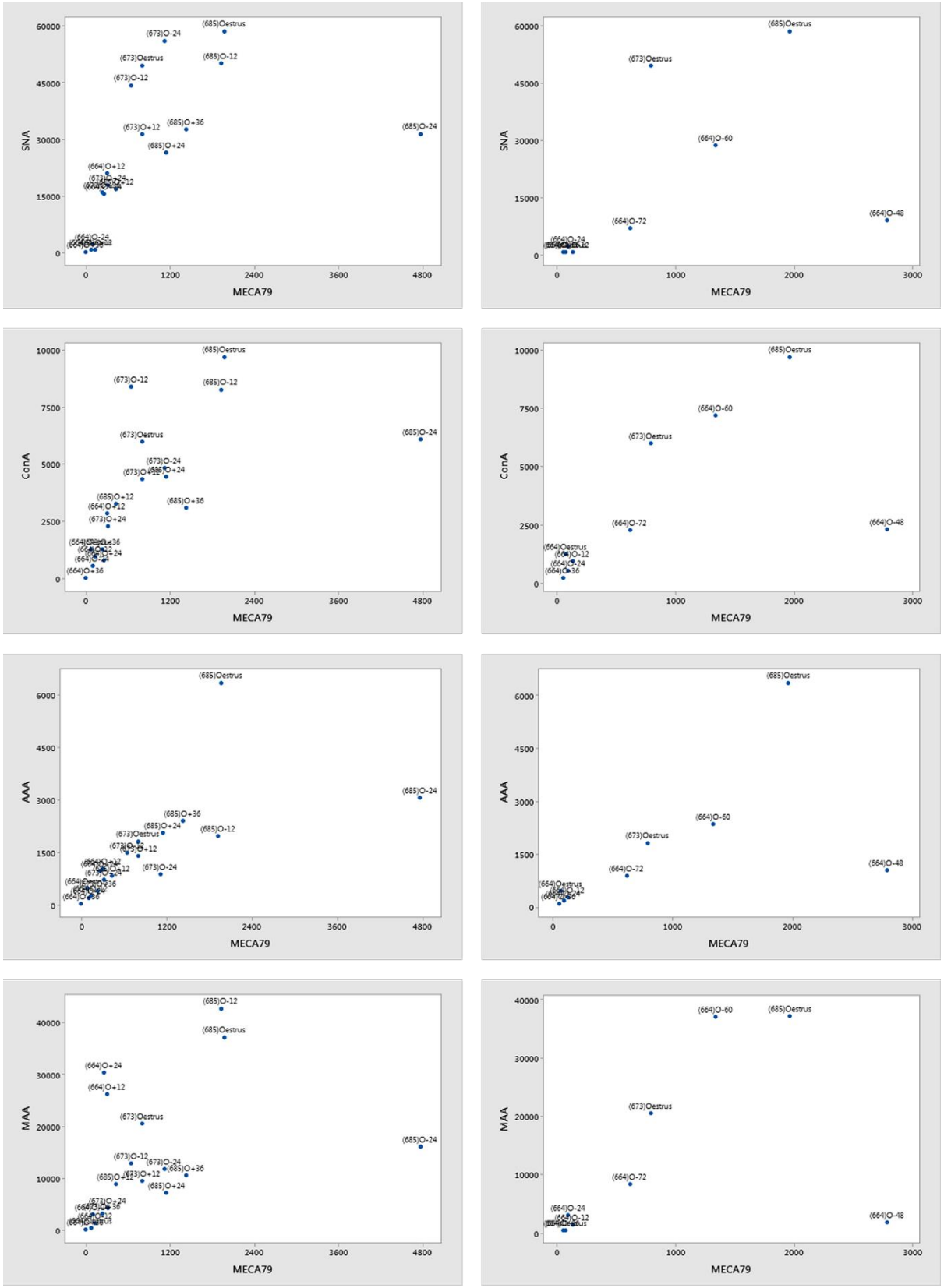

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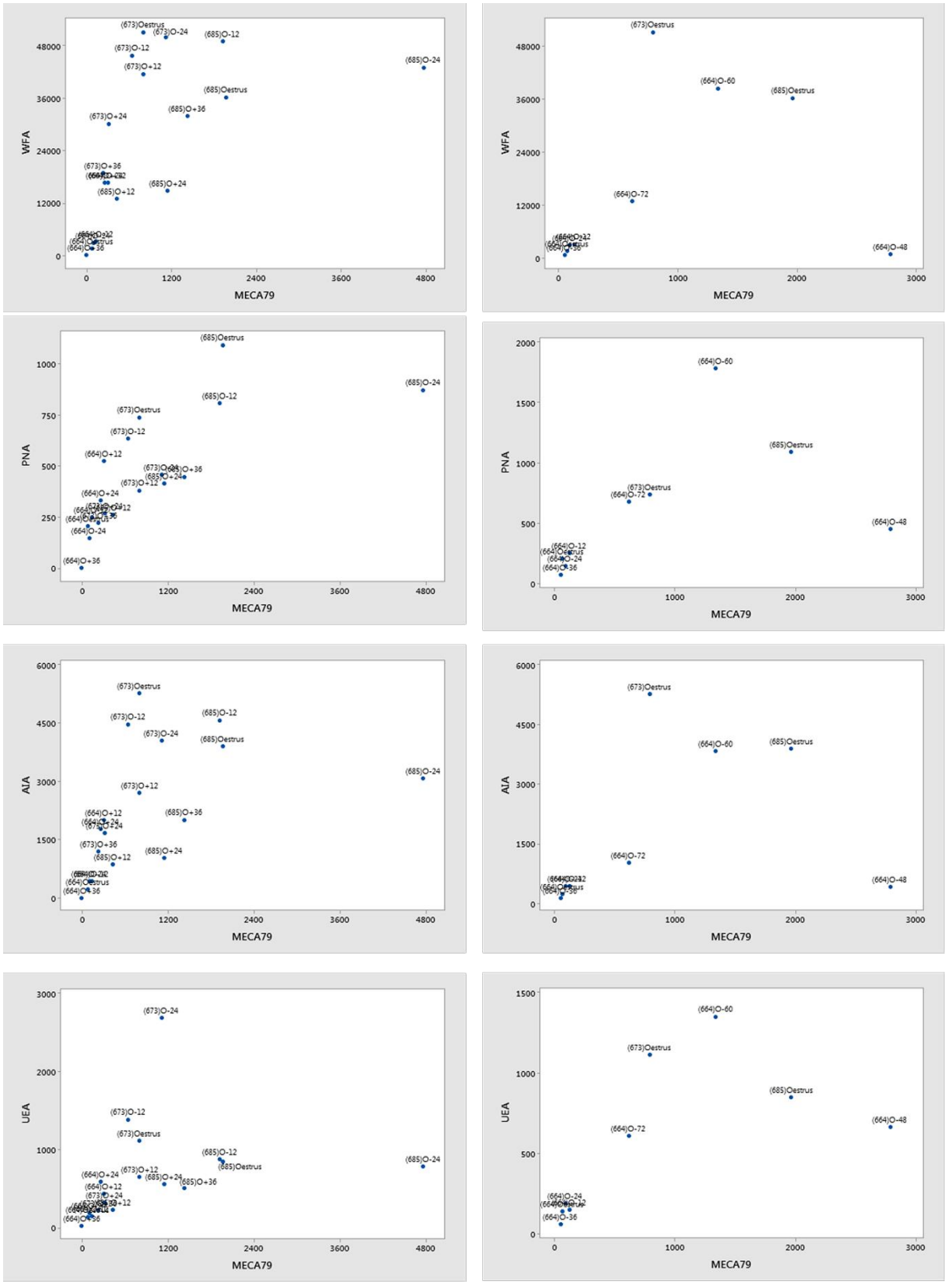

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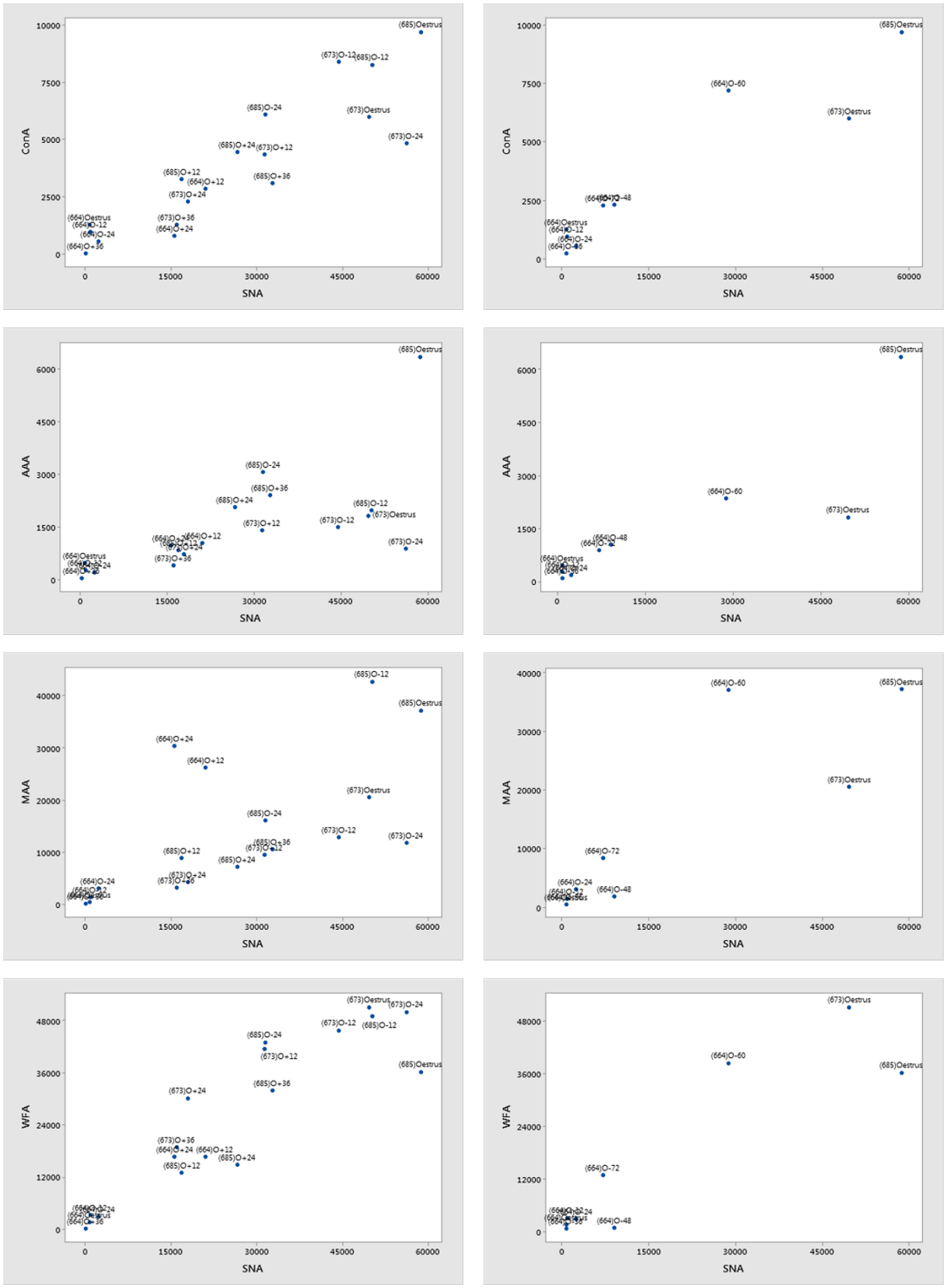

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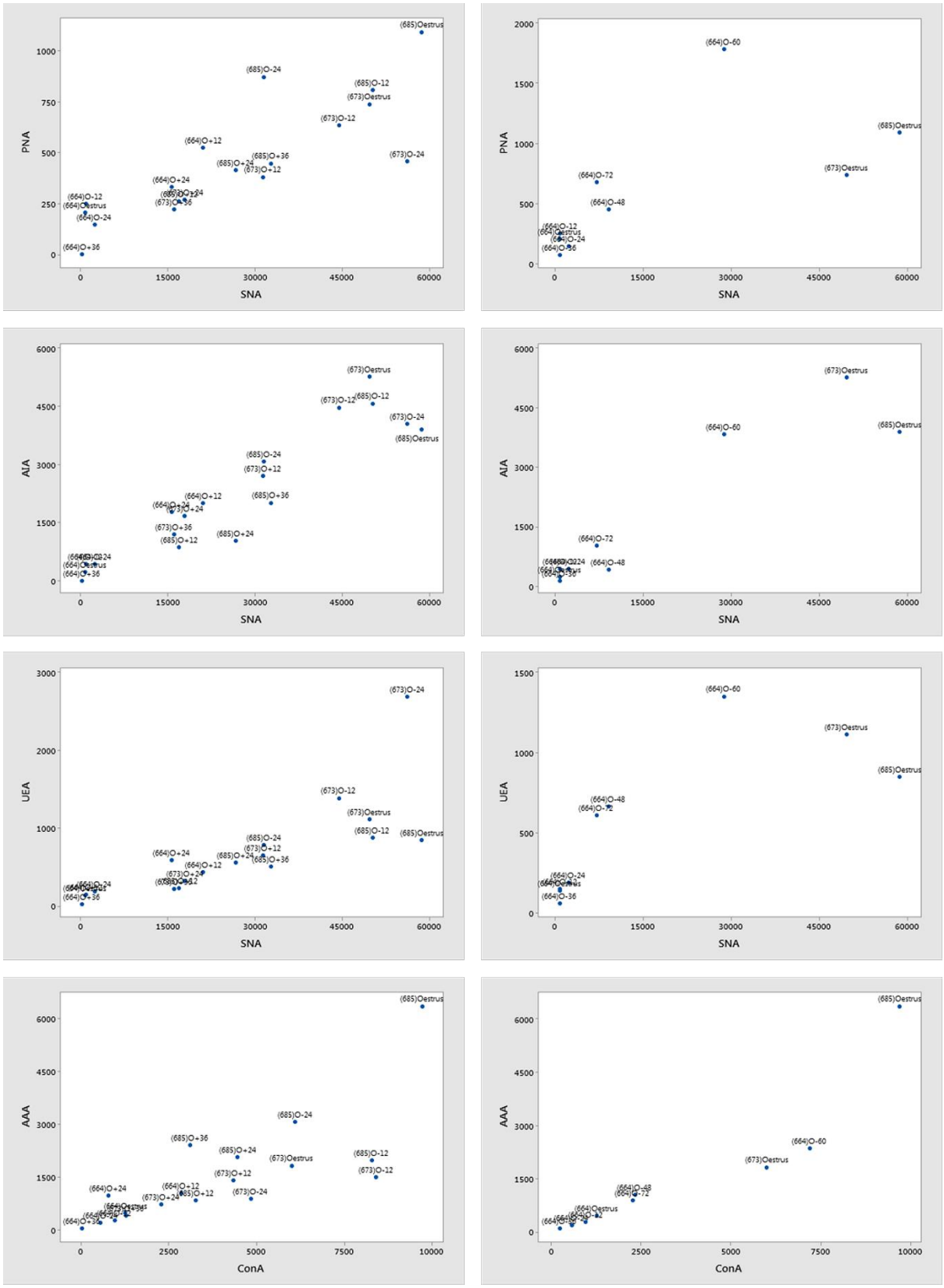

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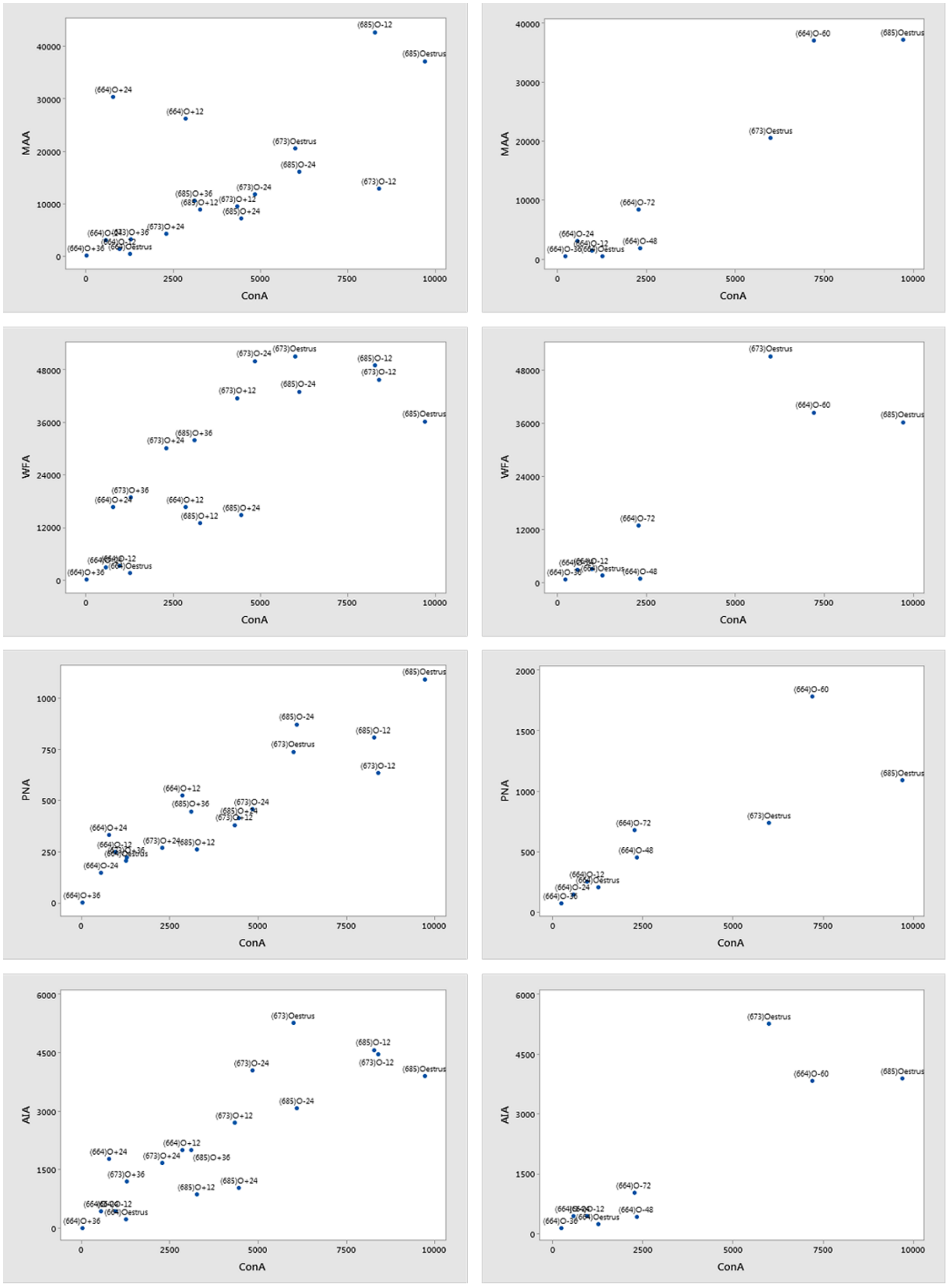

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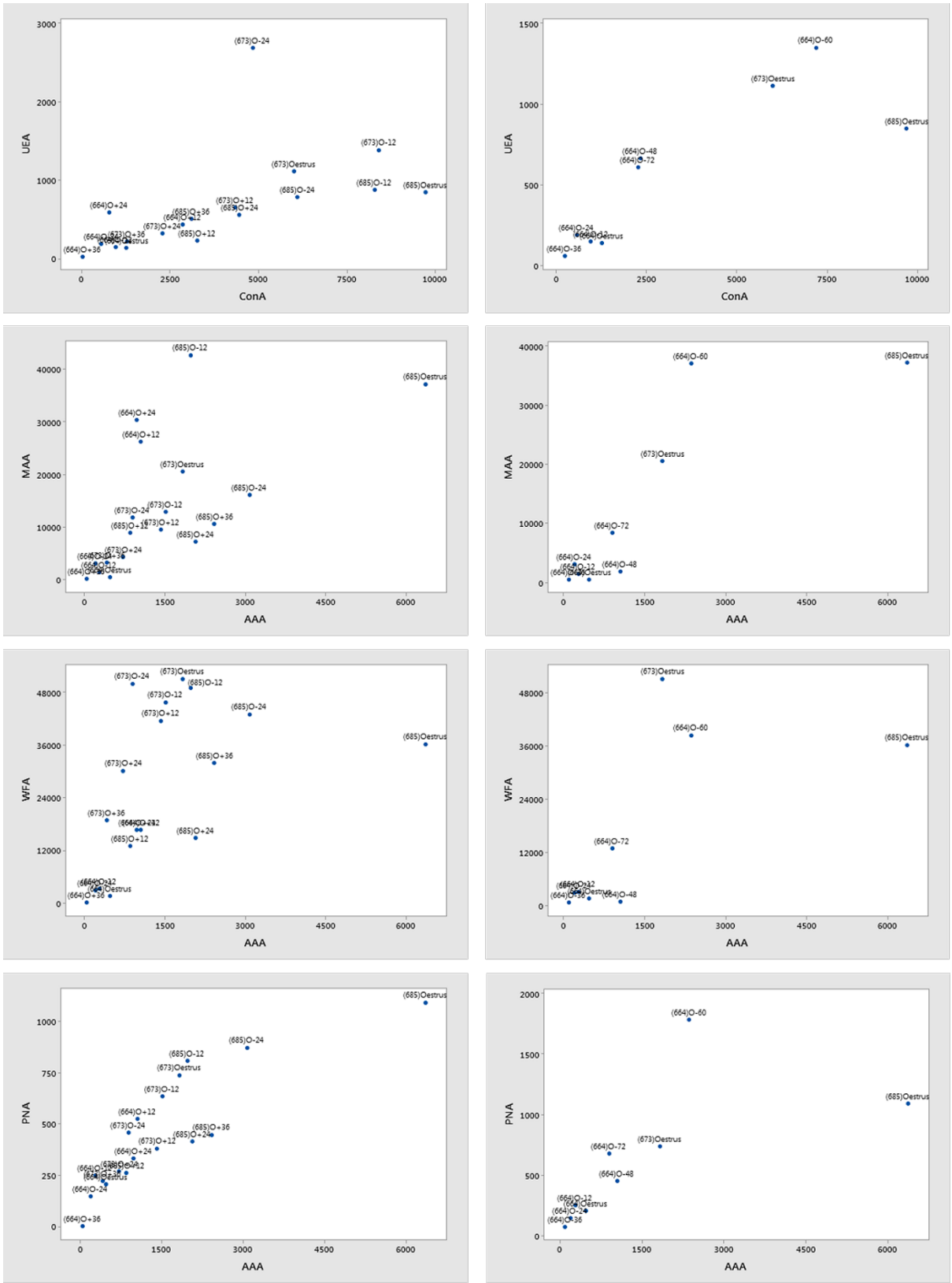

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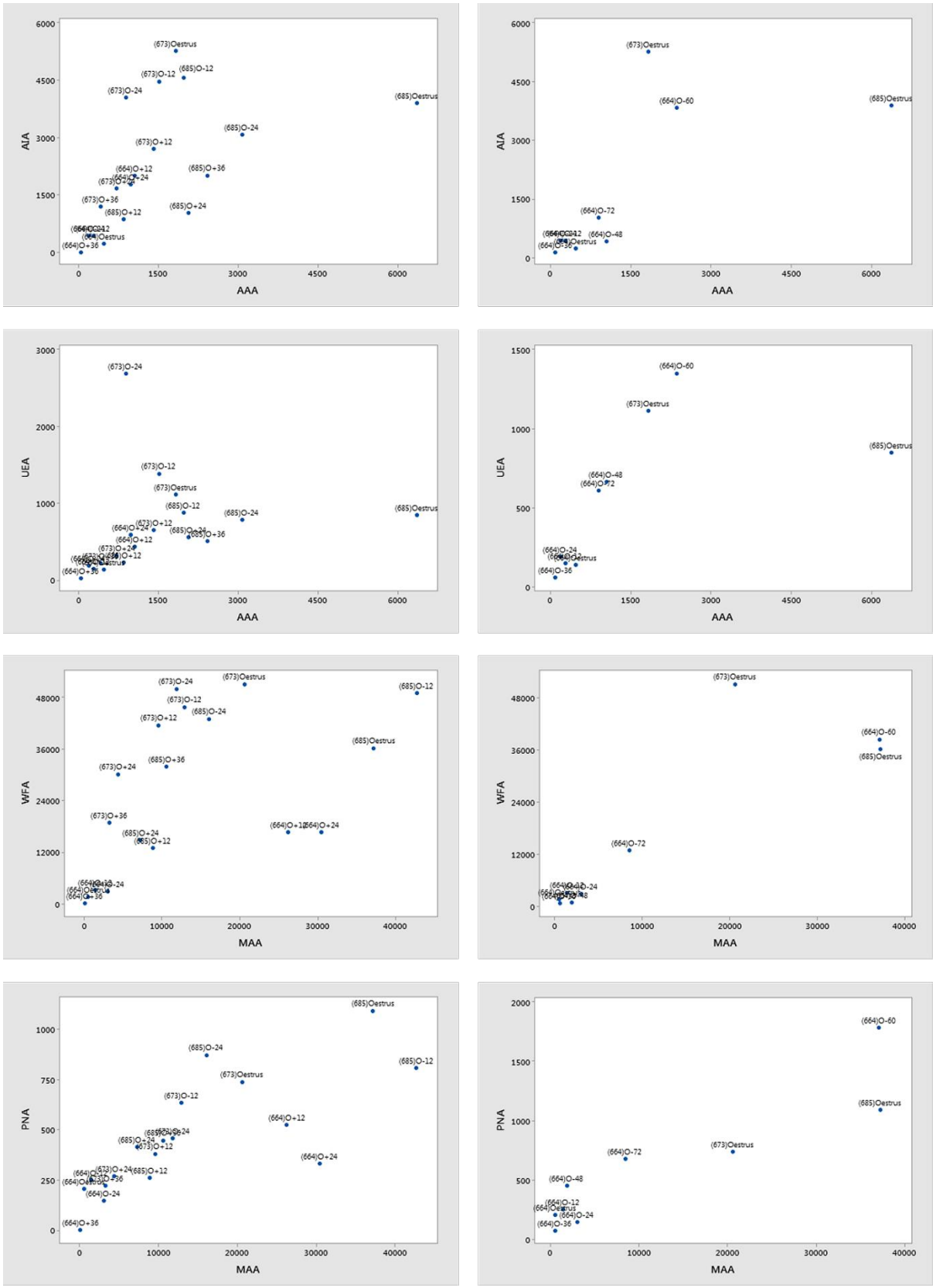

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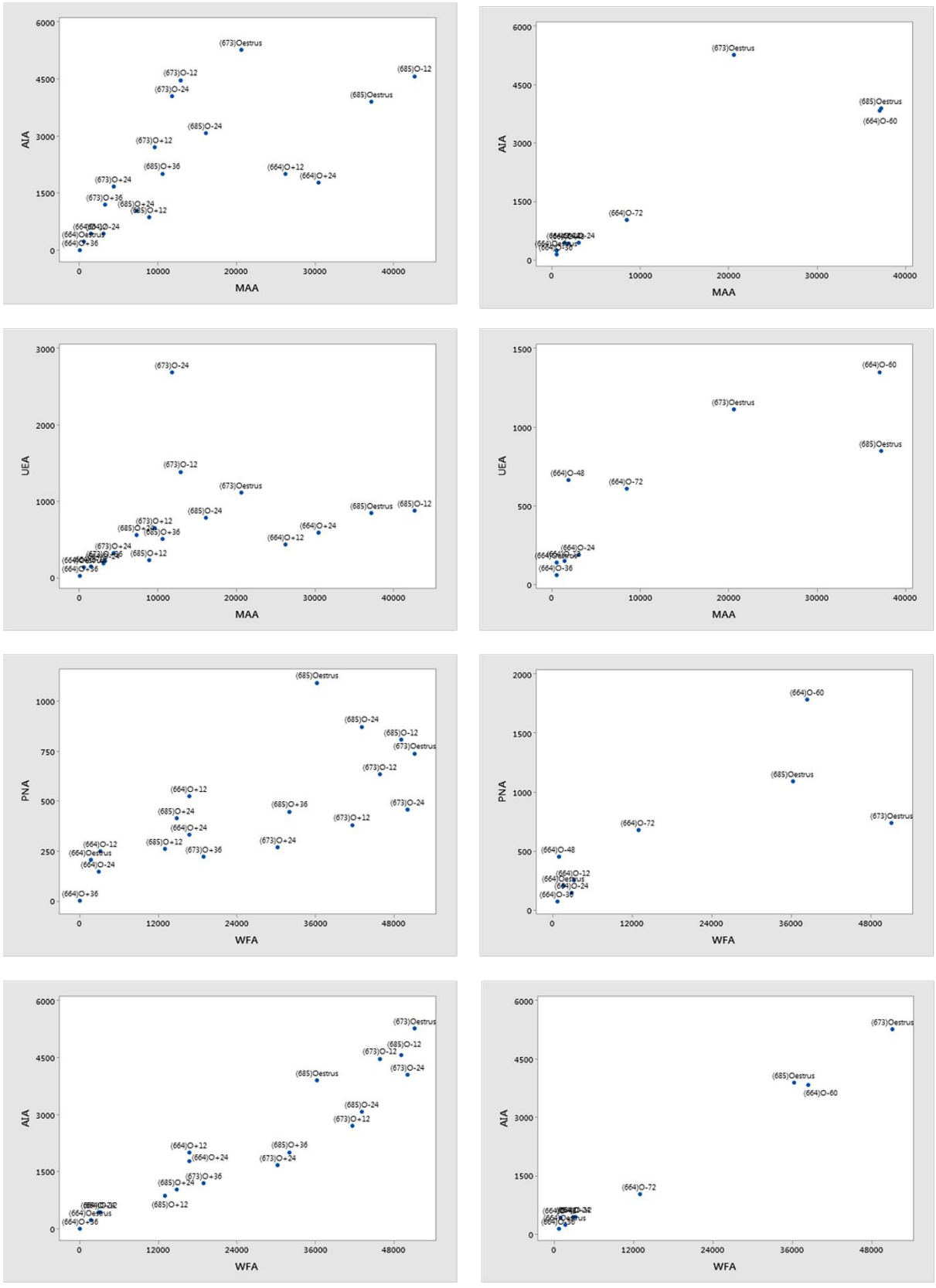

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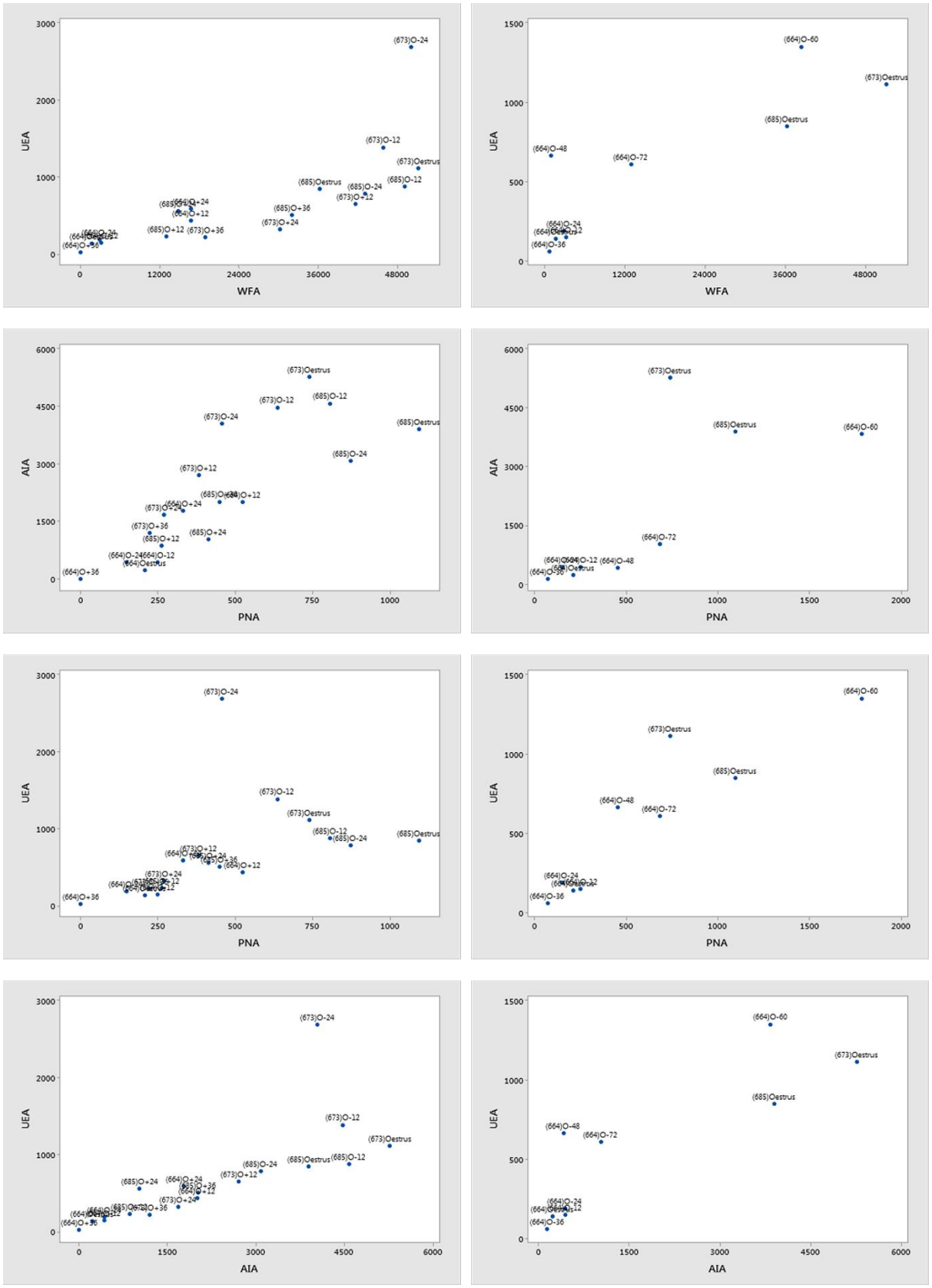

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