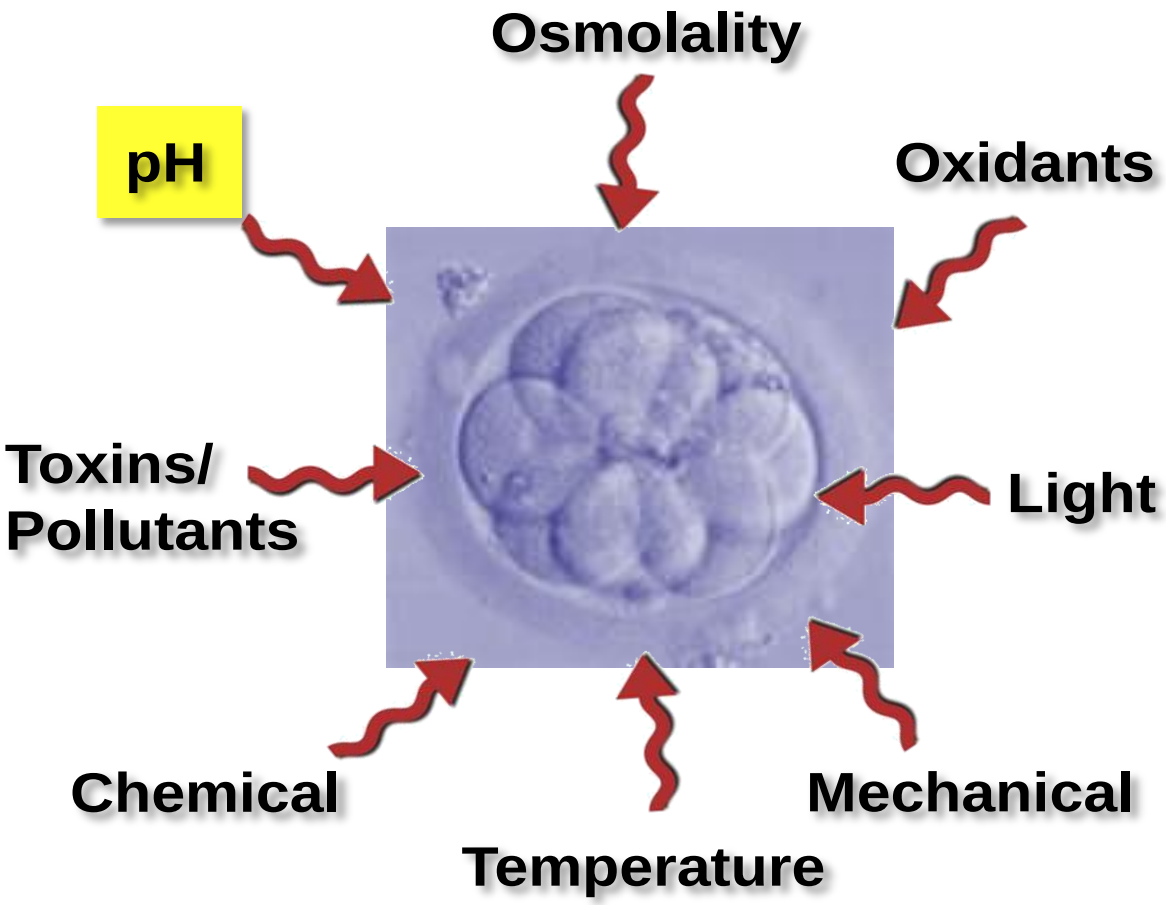
MHM™ :

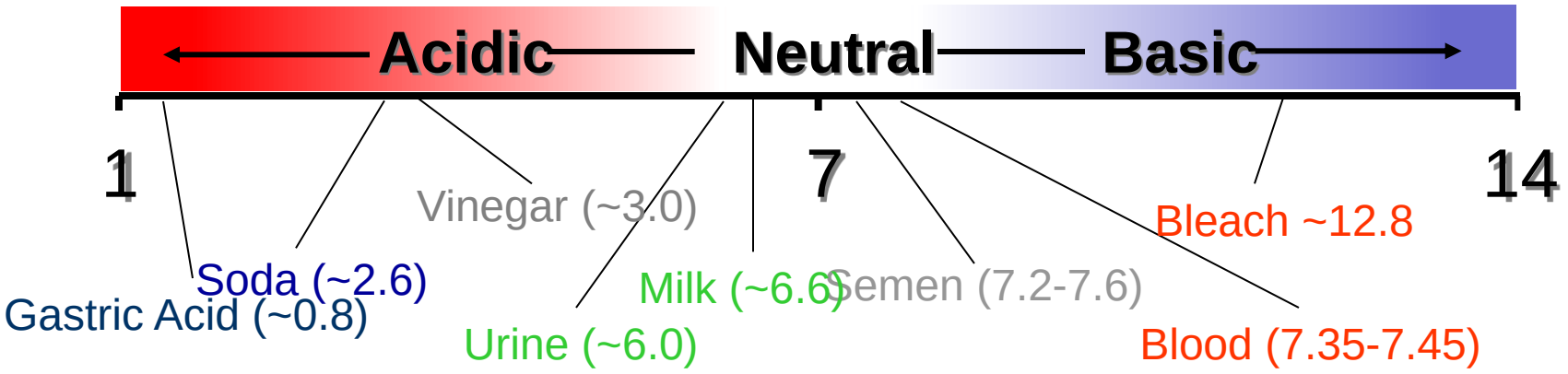
A Unique and Improved IVF Handling Media

In Vitro Stressors



Reduce stress to improve embryo development and ART outcomes

What is pH?

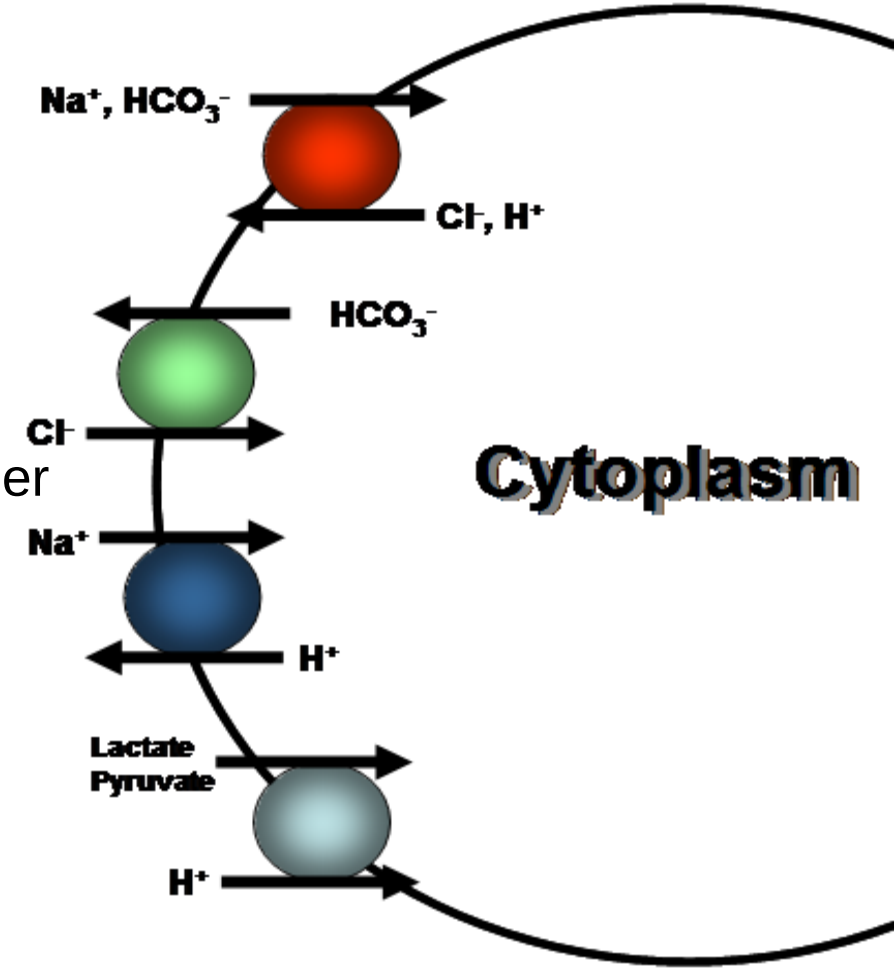


- Acids increase the concentration of hydrogen ions
- Bases decrease the concentration of hydrogen ions

pH is the measure of $[H^+]$

Internal pH (pHi)

- Cells contain pHi regulatory mechanisms
  - HCO_3-/Cl- exchanger >7.2-7.3
  - Na^+/H^+ antiporter <6.8
  - Na+ dependent HCO_3-/Cl- exchanger <7.0



- **pH_i follows external pH (pHe) of media initially**

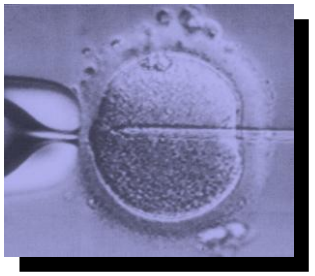
pHi and the Embryo

- Slightly raising or lowering pHi for 3hrs results in disorganization of mitochondria and actin cytoskeletal elements (Squirrell et al. 2001)
  - Regulate development and chromosome dynamics
- Raising pHi 0.09-0.15 for 4hrs significantly changes metabolism (Lane et al. 2000)
  - Metabolism is correlated with developmental competence
- Lowering pHi ~0.15 affects blastocyst development and resulting fetal size (Zander-Fox et al. 2010)

pH and ART

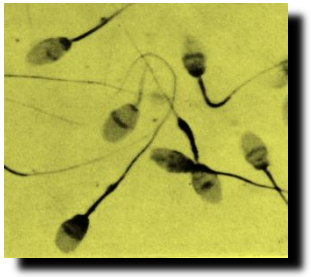
- Denuded mature oocytes lack robust pH_i regulatory mechanisms
  - Activated ~6h after fertilization

  (Phillips et al. 1998, 2000, 2002)



- Cryopreserved/thawed embryos have reduced ability to regulate pH_i
  - ~3h recovery (Lane et al. 2000)

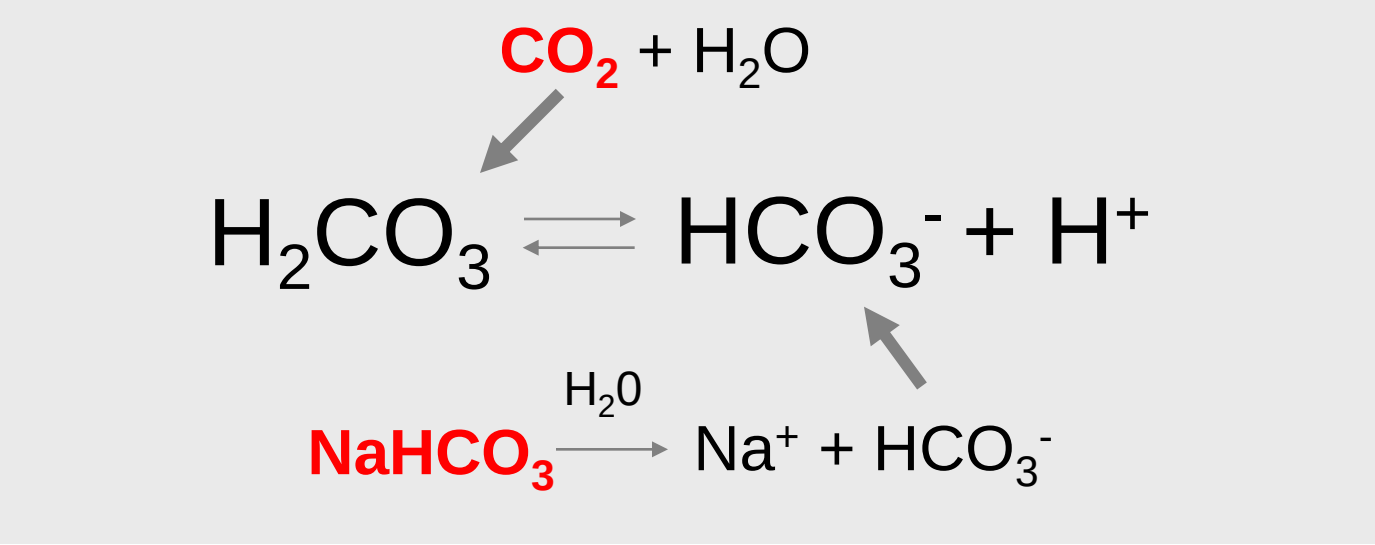
- Sperm pHi and function are influenced by pHe (Hamamah et al 1996)



Proper and stable pHe is crucial

External Media pH (pHe)

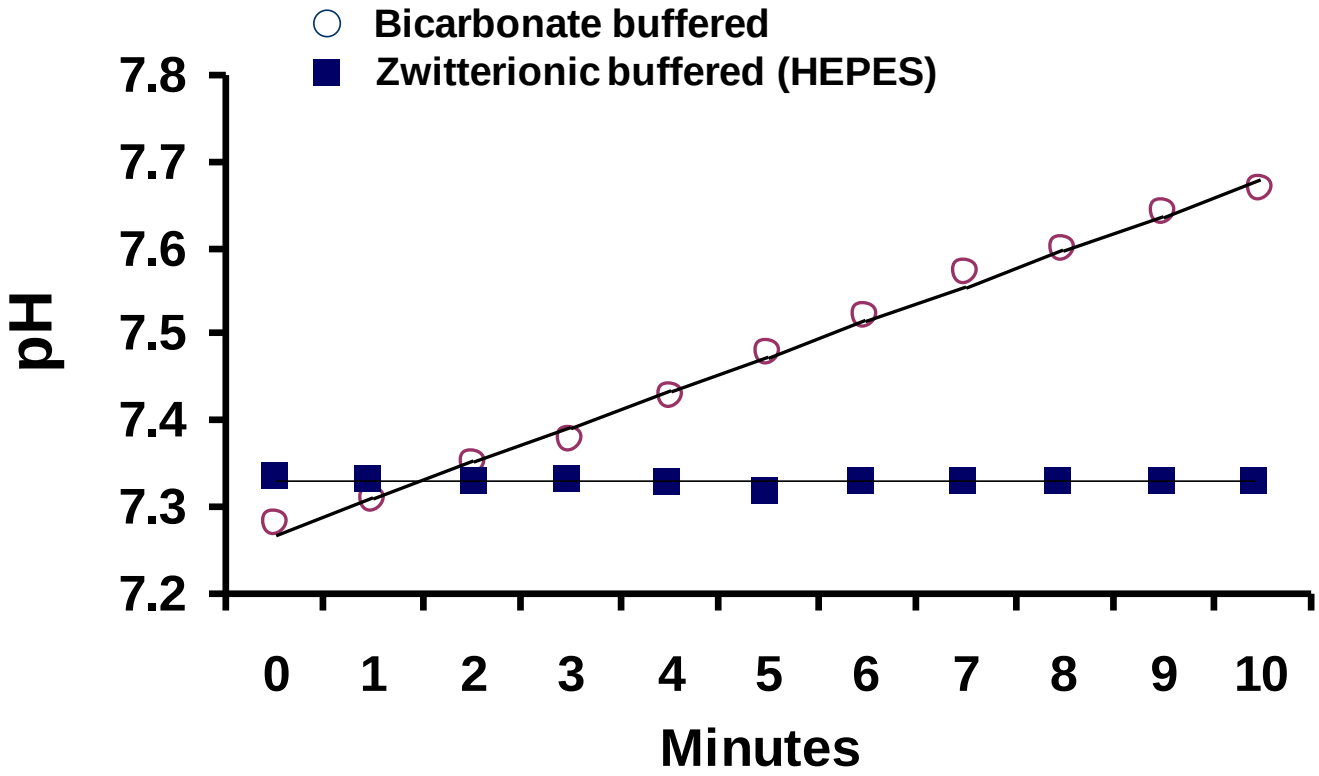
Incubator vs. Media



How do we control pHe outside the incubator?

IVF Handling Media

Media that uses reduced bicarbonate concentration and includes a zwitterionic buffer, like HEPES or MOPS, to maintain pHe outside the incubator



Importance of Handling Media

- Brief exposure to inappropriate handling media can significantly reduced embryo development

-Hamster (Escriba et la. 2001)

-Rabbit (Farrell & Bavister 1984)

-Cow (Palasz et al. 2008)

-Mouse (Gardner & Lane 1996)

-Human (Morgia et al. 2006)

Common Concerns with Buffers

- Buffers, like HEPES, are toxic
- Injection of buffers may alter pHi (Morgia et al. 2006)
- HEPES and MOPS block Cl^- channels and may inhibit blastocyst development (Yamamoto and Suzuki, 1987, Butler et al., 1988).
- Cell specific sensitivity to particular buffers (Eagle 1971)
- Concentration dependent side-effects of buffers(Downs & Mastropoki 1997, Iwasaki et al. 1999)

Though many of the concerns are unfounded, this presents an opportunity to develop an improved handling medium

Objective

- Develop a unique and improved IVF handling medium
  - Accomplish with minor modifications to an already accepted medium to facilitate acceptance

1) Reduced buffer concentration
2) Improved buffer selection
   -Buffering capacity (pKa)
3) Inclusion of select amino acids

Reduced Buffer Concentration

Concerns with Buffers

- Detrimental effects described with some buffers may be concentration dependent (Downs & Mastropoki 1997, Iwasaki et al. 1999)
    - Increasing buffer from 20 to 25mM prevented pharmocologic inhibition of oocyte maturation
    - Increasing buffer above 35mM increased pig embryo degeneration

Buffer Concentration

- ~2x the concentration of zwitterionic buffer as bicarbonate is sufficient to stabilize pHe (Freshney 1983)

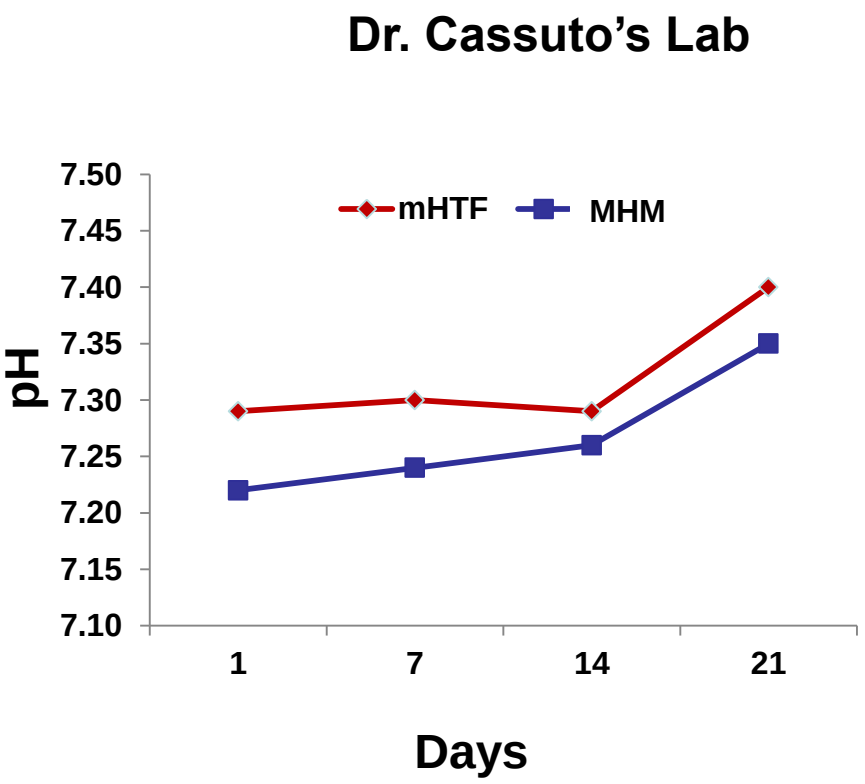
- Most IVF handling media contain ~21mM buffer
  - 5.25X conc. of 4mM bicarb

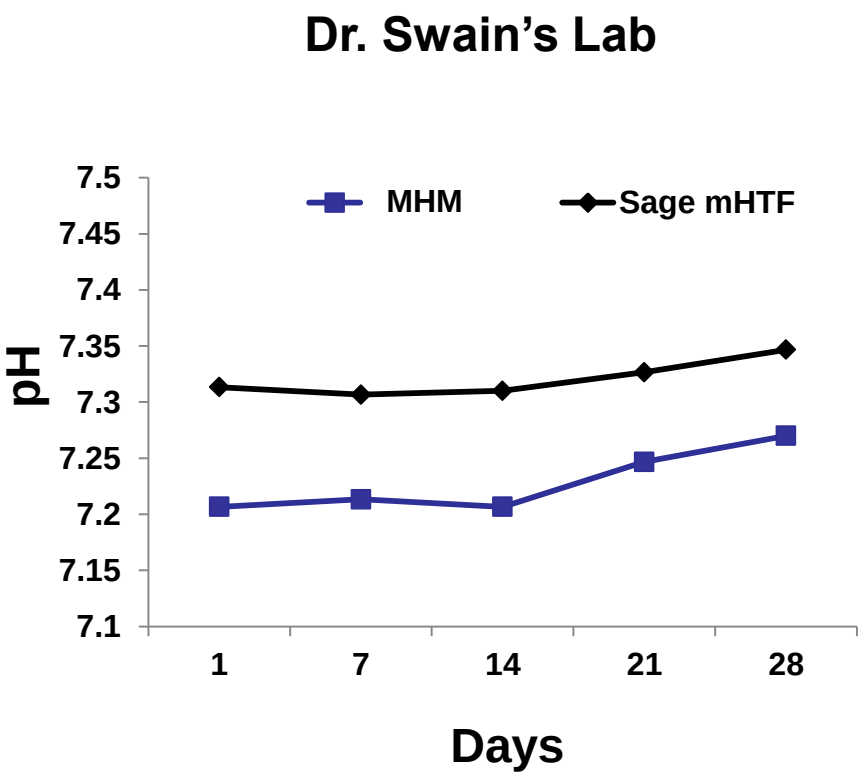
How did we arrive at current formulations?

Objective

Determine if reduced buffer concentration in IVF handling media maintains pHe stability and supports embryo development

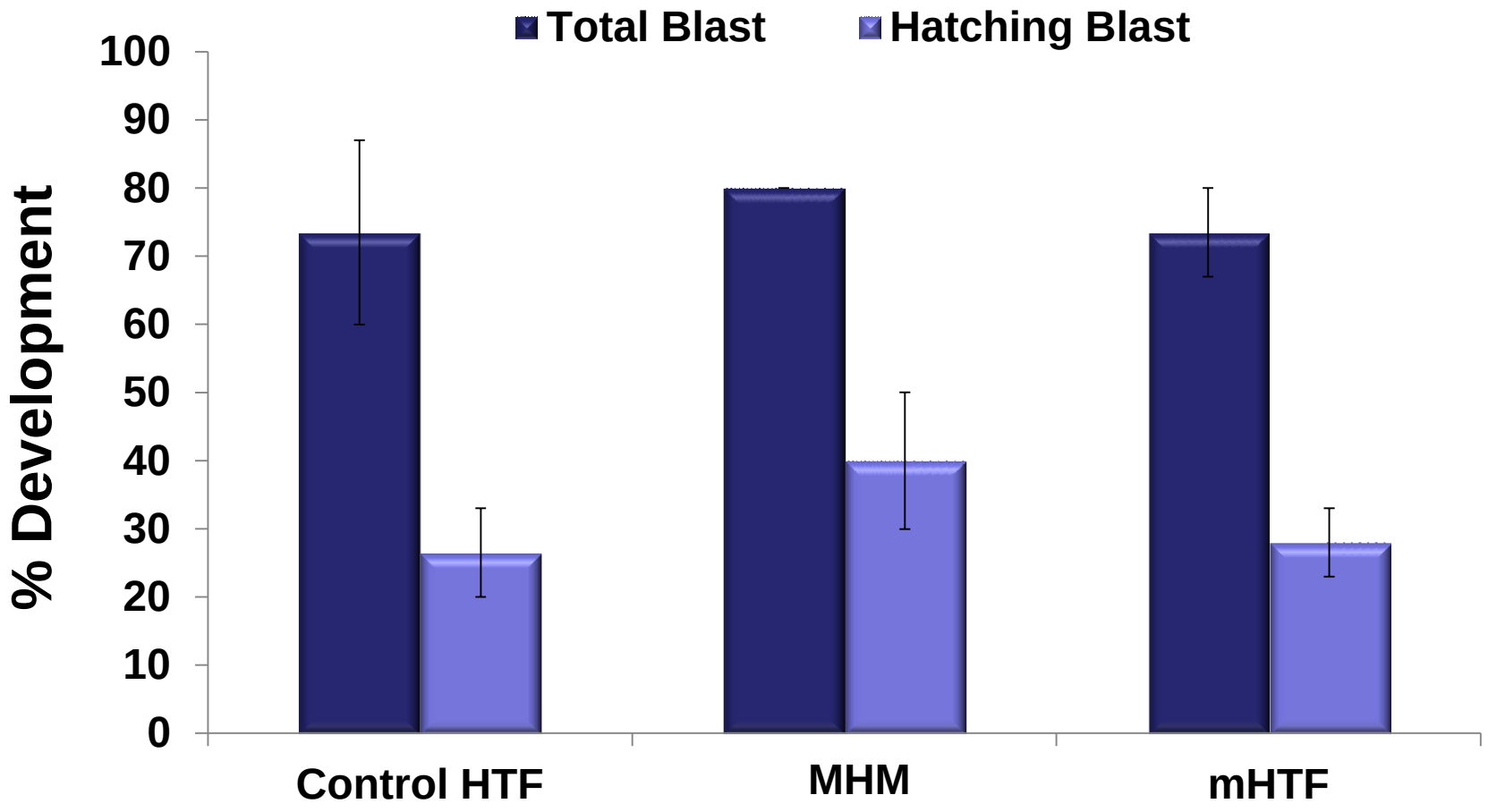
MHM™ - pH Stability





MHM™ - MEA Development

2 Beta Site Labs – 2 hr MHM Exposure – 96h of culture in incubator



Custom Combination Buffering System to Optimize Buffering Capacity Over a Range of Temperatures

Objective

Formulate a dual buffered system that offers improved buffering capacity over a range of temperatures compared to current single buffered media containing only HEPES or MOPS

Buffer Selection

- Buffers are selected based on ability to support cell growth
  - Not all buffers are compatible with all cell types

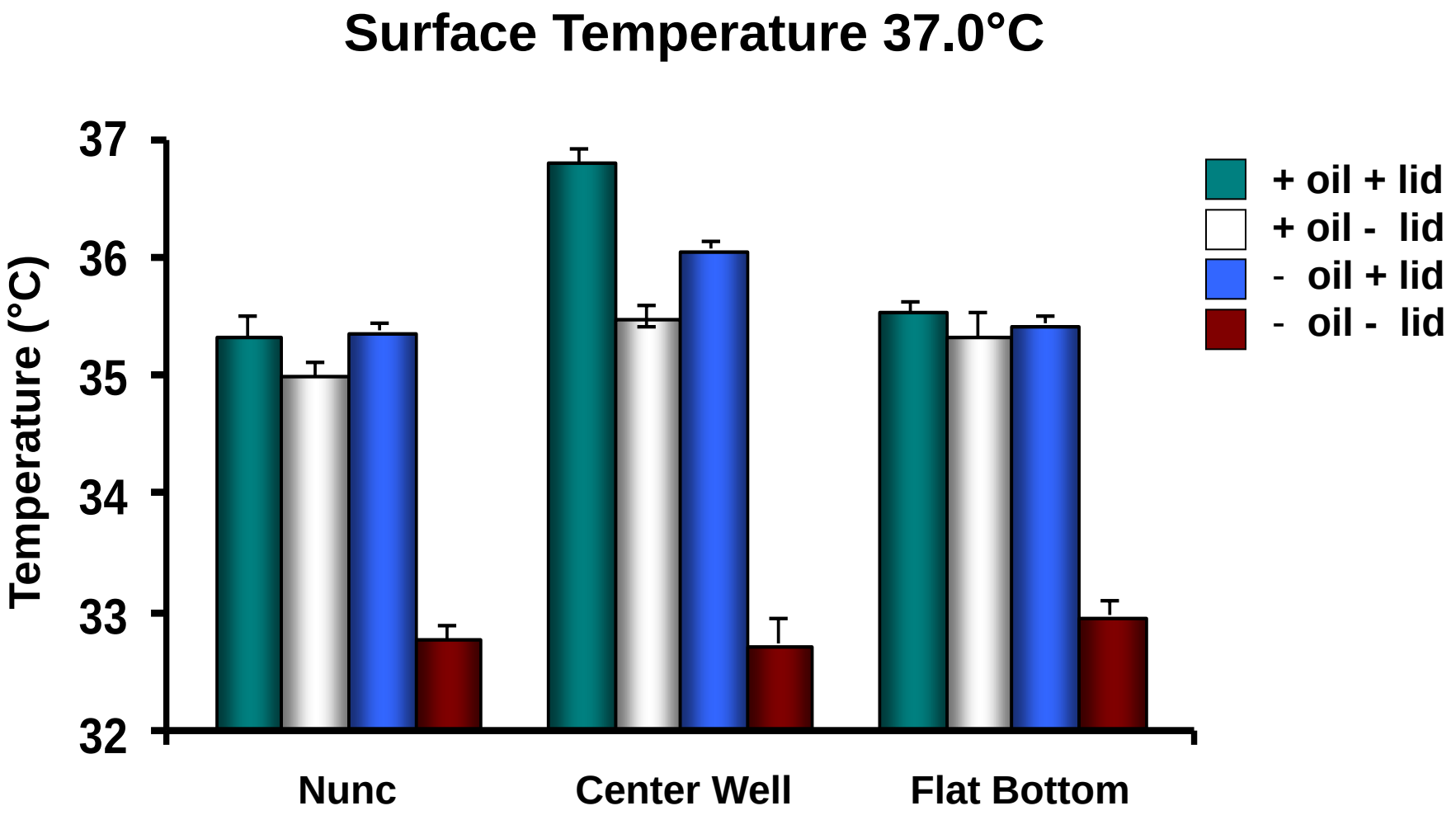
- Compatible buffers are then chosen based on their maximal buffering capacity…or ability to maintain a specific and stable pHe
  - Maximal buffering is indicated by a buffer's pKa value
  - Maximal buffering is obtained when pKa is equal to the the desired pHe (7.2-7.4 in IVF labs)

Maximal Buffering: $pH = pK_a$

Buffers & pKa

Common Name	pK_a at 20°C
TAPSO	7.7
DIPSO	7.6
HEPES	7.55
TES	7.5
Phosphate*	7.21
MOPS	7.20
Carbonate*	6.38

Temperature in the lab

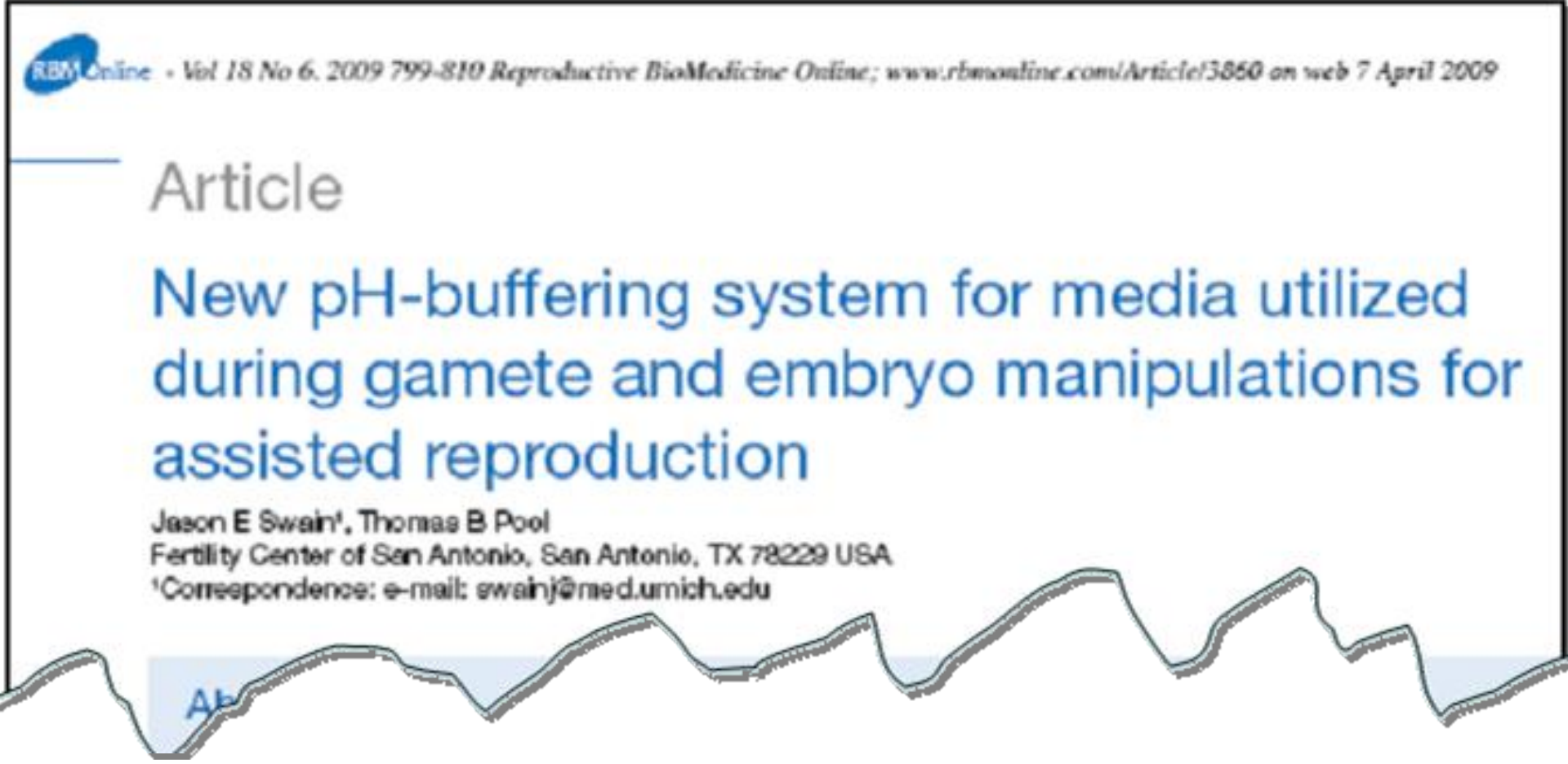


Temp will vary throughout the lab, and even depending on the dish/volume used

Buffers & pKa

Temperature Impacts Buffering

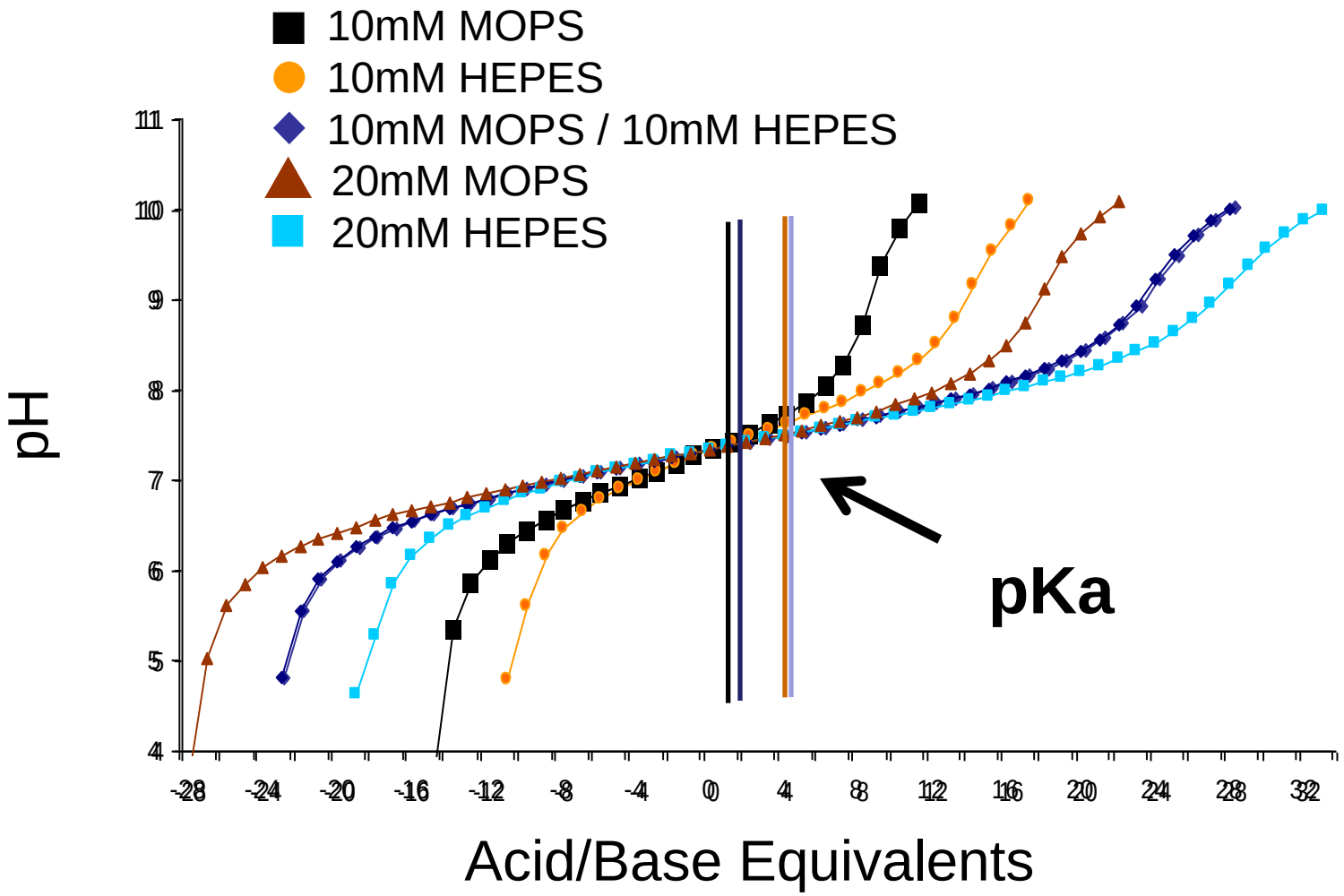
Common Name	pK_a at 20°C	pK_a at 37°C
TAPSO	7.7	7.39
DIPSO	7.6	7.35
HEPES	7.55	7.31
TES	7.5	7.16
Phosphate*	7.21	7.19
MOPS	7.20	6.95
Carbonate*	6.38	6.30



Combine zwitterionic buffers

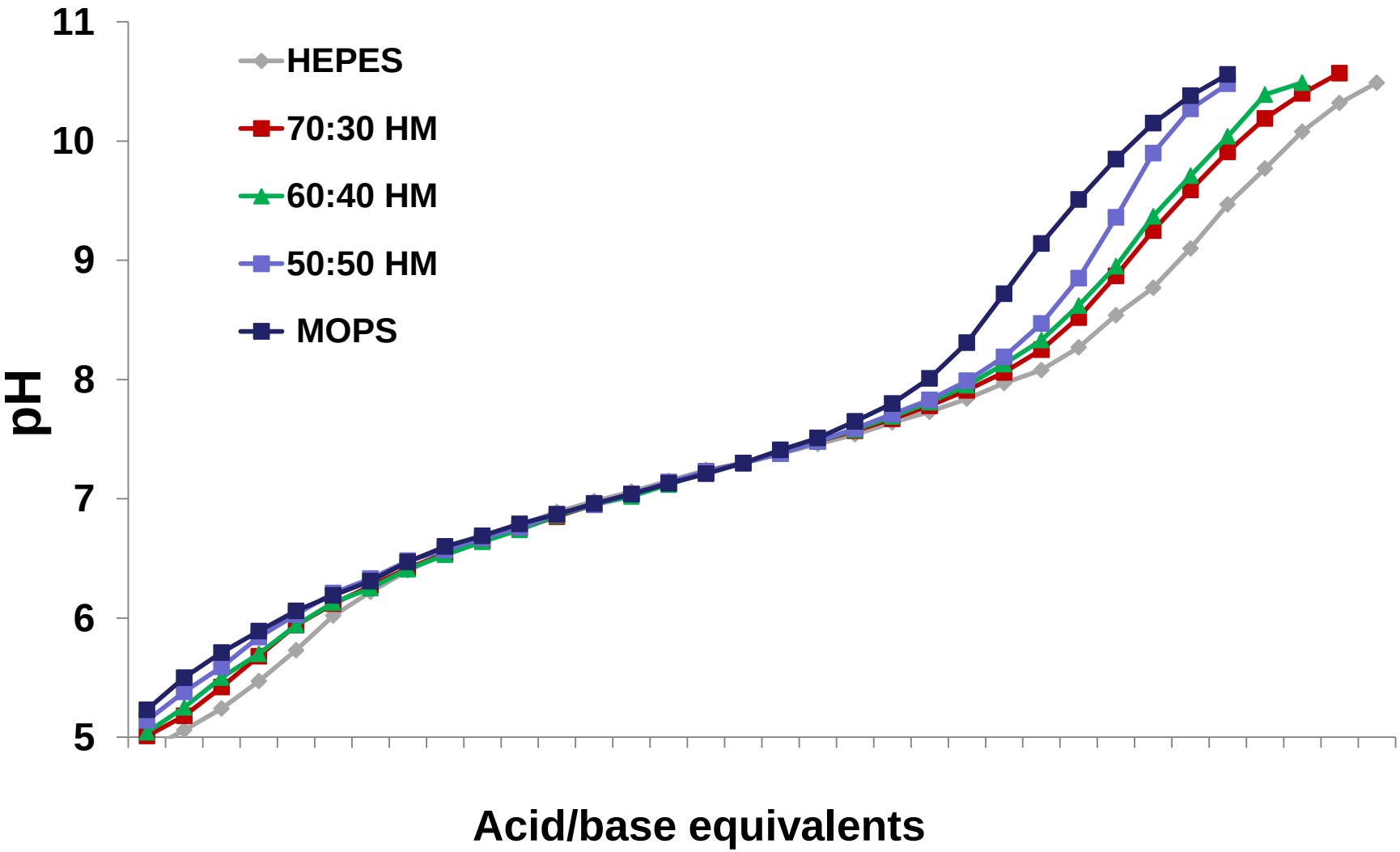
- To avoid concern with elevated concentration and possible toxicity:
- To optimize pH buffering capacity (pKa) considering temp

Combination Buffers



Swain et al. 2009

Combination pH Buffering



Conclusion

- Varying ratios of HEPES and MOPS allows for adjustment of optimal buffering and allows for the creation of a unique handling media that provides optimal buffering over the range of temperatures encountered in and IVF lab

Amino Acids and IVF Handling Media

Objective

- **Examine the effect of various amino acids and their combinations on embryo development when included in an IVF handling medium, focusing on amino acids that could be included in a universal handling media suitable for gametes and all embryo stages**

1) NEAA
2) Glutamine
3) Glycine
4) Taurine

Amino Acids

- Amino acids act as metabolic substrates, osmolytes and regulators of pHi (Lane 2000)

- Absence of amino acids in handling media resulted in significantly decreased blastocyst formation in mouse (Gardner & Lane 1996)

All media should contain some assortment of amino acids

Amino Acids

- Some amino acids are beneficial, while others are detrimental – dependent upon concentration and cell type/stage
  - Cleavage stage mouse embryos benefit from inclusion of NEAA (glutamine), while EAA are beneficial post-compaction (Gardner & Lane 1993, 1994, 1997a,b)
  - Glycine, taurine and glutamine found most beneficial for hamster embryos (McKiernann et al. 1995)
    - Taurine acts as an osmolyte and is beneficial for human embryos (Dumoulin et al. 1997, Dawson & Baltz 1997)
    - 1 report of benefit of glutamine for human embryos grown in glucose-free media (Devreker et al. 1998)
    - Glycine is a potent osmolyte and transporter identified in human embryos (Hammer et al. 2000)

Amino Acids - Results

- After numerous experiments:
  - No significant benefit of NEAA alone or in combination with other amino acids were found at varying concentrations
  - No significant benefit was found when including glutamine, alone, or in combination with other amino acids
  - Though not significant, taurine and glycine supplemented in combination gave slightly higher rates of embryo development compared to other treatments
    - Are included in most IVF medium, have known functions

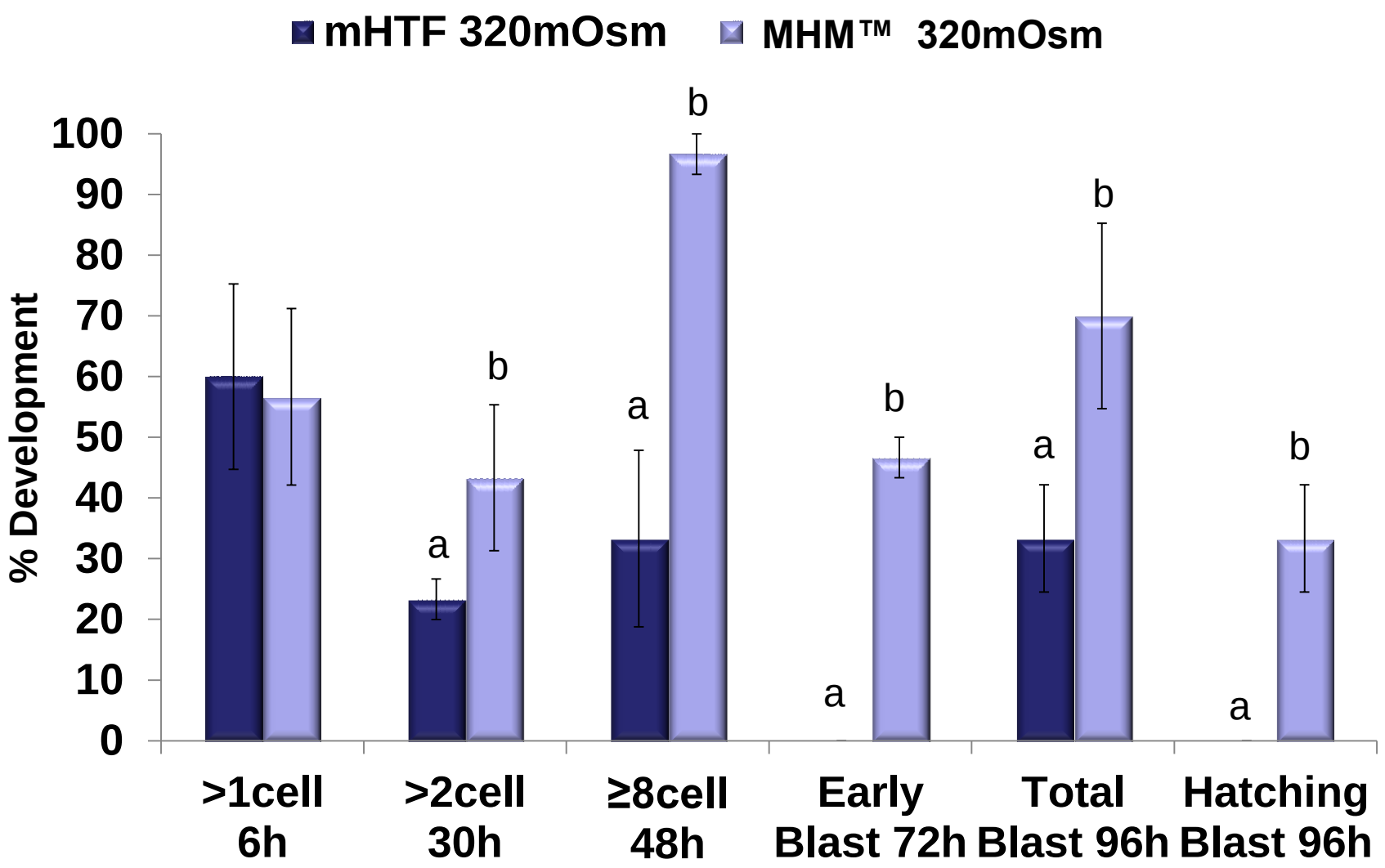
Why Exclude Glutamine?

- Glutamine impacts glucose metabolism (Chatot et al. 1990, Du & Wales 1993)
  - Trying to avoid metabolic perturbations – saw no benefit in our study
- Glutamine is labile in culture and can for harmful ammonia – this necessitates use of dipeptide forms
  - Dipeptides may not function as optimally as individual amino acids (Swain et al. 2011)
- Glutamine utilizes the same transporter as glycine for osmoregulation - redundant
  - Glycine has been shown to inhibit glutamine transport in post compaction mouse embryos, likely because both use the same GLYT1 transporter (Richards *et al.*, 2010)

Rationale for Amino Acid Selection

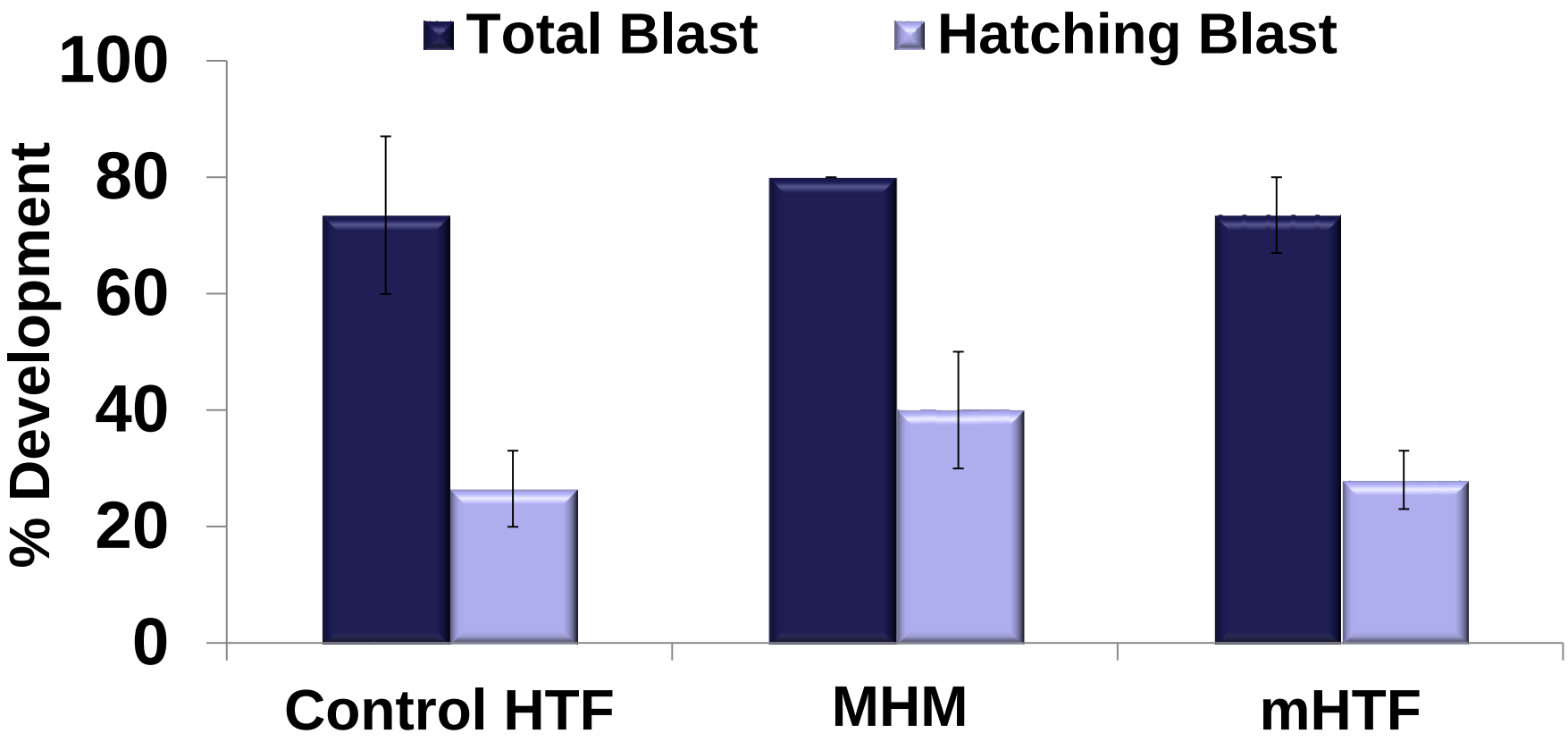
- NEAAs don’t appear to be beneficial in the context of the basal media used
  - Why include unnecessary amino acids and risk potential negative side effects like ammonia buildup?
- No significant benefit of glutamine observed in context of our basal medium and potential drawbacks exist
- glycine + taurine appear slightly beneficial
    - Known/proven osmolytes and/or benefit in human embryos

MHM™ - Osmo Protection

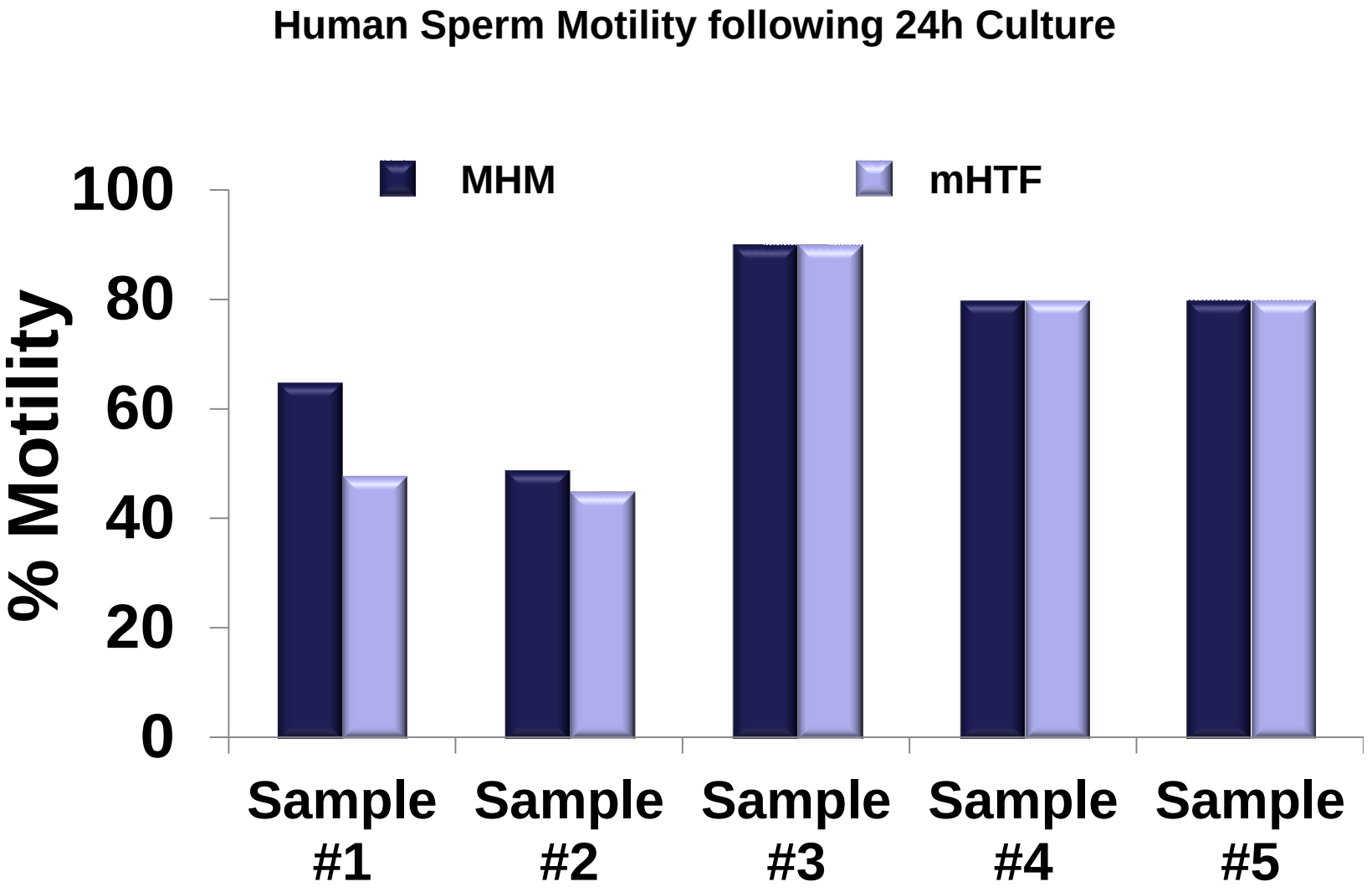


MHM™ Clinical Testing

MHM™ 1-cell MEA



MHM™ - Clinical ISCI Data



MHM™ - Clinical ISCI Data

Rationale for Testing with ICSI - Oocyte is most pHe sensitive cell stage
- Most invasive use of buffered media and most likely scenario to see impact